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Surface Specific Peptide Immobilization on Radiografted Polymers as Potential Screening Assays for Antiangiogenic Immunotherapy

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Abstract:

Angiogenesis is a key process of cancer development and metastasis. Its inhibition is an important and promising strategy to block tumor growth and invasion. One of these approaches, based on antiangiogenic immunotherapy, is the recognition of a specific region of an angiogenic growth factor, called VEGF-A, by monoclonal antibodies. Thus, we aimed to design a novel assay to screen potential monoclonal antibodies directed against VEGF-A. In a first approach, we chose to perform covalent coupling of angiogenesis active cyclopeptides onto biocompatible thermoplastic transparent PVDF films and to fully characterize the chemical structure, the surface state and the biochemical properties of the synthesized devices. Electron beam radiation created radical sites on PVDF films without adding any toxic chemicals. These primary radicals and some induced peroxides were used as initiators for acrylic acid polymerization. Under our experimental conditions, surface grafting was favoured. Functionalization of PVDF-g-PAA films with peptides *via* a spacer arm was possible by performing two subsequent coupling reactions. EDC was used as coupling agent. Spacer arm saturation of the film surface was achieved for 25 mol% yield meaning that one spacer arm on 4 carboxylic acids were covalently bound. Peptide immobilisation resulted in binding 10 times less leading to a final 3 mol % yield. Binding densities are governed by their individual space requirements. Each chemical step has been followed by FTIR in ATR mode, NMR using HR MAS technique and XPS. From XPS results, a layer of peptide covered PVDF-g-PAA film surface. The amounts of covalently immobilised peptide were determined using indirect UV spectroscopy on supernatant reaction solution. Yields were correlated with High Resolution NMR results. The peptide/antibody recognition validated our system showing the conservation of peptide tridimensional structure with a positive response to specific antibodies. Because of the covalent protein linkage to PVDF films, a simple cleaning with immunoaffinity chromatography buffer allows the films to be reused.

1. Introduction

Angiogenesis is a key process of cancer development and metastasis. Angiogenesis is the process by which new capillaries sprout from pre-existing blood vessels [1]. Then inhibition of angiogenesis is an important and promising strategy to block tumor growth and invasion. Several approaches could be used for inhibiting angiogenesis as blocking interactions between angiogenesis growth factor and their receptors, inhibiting intracellular pathways... There are currently more than 30 angiogenesis inhibitors (VEGF and VEGF receptor antibodies, kinase inhibitors ...) in clinical trials and a multitude of promising new candidates are under investigation *in vitro* and in animal models.

Immobilizing such peptides onto a solid support may be of great interest for the design of reusable assays in order to screen potential monoclonal antibodies for anti-angiogenic therapy. To date, general screening of antibodies has mainly used ELISA assays. These common assays present some disadvantages: (i) epitope sites of adsorbed biological molecules could not be completely accessible for antibodies (ii) adsorption is an aspecific process which can induce contamination and increase artefacts

(iii) the tests are not reusable which increase the cost of the assays with synthetic targets. To overcome these drawbacks, we focused our approach on the synthesis of a novel device based on a polymeric support which presents epitope sites in optimum accessibility. Moreover, biological molecules of interest are covalently bound to the support to decrease possible contamination and to undergo harsh chemical conditions required to break antibody-antigen complex.

Several growth factors have been involved in angiogenesis regulation. Among them, VEGF-A (Vascular Endothelial Growth Factor) is a key regulator of both physiological and pathological angiogenesis[2]. Studies have demonstrated that inhibition of VEGF-A activity on its type 2 receptor (VEGFR2 or KDR) results in suppression of tumour growth *in vivo* [3]. Some of the important residues for the interaction between VEGF-A and its receptor have been investigated. These residues are clustered within region 79-93 of VEGF forms a β -hairpin. This amino acids sequence and its structural conformation could allow to access specific antiangiogenic therapies. Based on these considerations, we designed a series of cyclic peptides in order to mimic the VEGF-A β -hairpin. Surprisingly, several cyclopeptides inhibit the VEGF-VEGFR2 binding [4]. They exhibited a good affinity for the KDR receptor and also inhibited endothelial cell proliferation and *in vivo* angiogenesis. One of the most potent among these original derivatives is a 17-mer cyclopeptide, called cycloVEGI (Vascular Endothelial Growth Factor Inhibitor).

Moreover, this novel compound is of a great interest for antiangiogenic immunotherapy. Indeed, the VEGF-A β -hairpin could be a potential epitope for antibody recognition. In order to screen novel specific VEGF-A β -hairpin antibodies, cycloVEGI appears to be a potential and promising target.

The present paper reports on the subsequent chemical steps from radiation induced graft polymerization to peptide immobilization. Acrylic acid (AA) was selected as part of the linker allowing peptide coupling onto the PVDF. This choice was guided because AA is a well-known monomer which polymerizes easily, to form a hydrogel. This hydrogel therefore supposed not to activate complement and allowed final PEG surface coupling to yield stealth biomaterials. Among various methods to graft

acrylic acid AA on PVDF [5-11], radiation grafting is a very suitable technique regarding biomedical applications. Indeed, there is no need of chemical initiators to polymerize AA and consequently less contaminant *in fine*. Spacer molecules being design to bring mobility to the peptides hydrophilic moieties were required.

Each step has been characterized by Fourier Transform InfraRed (FTIR) spectroscopy in attenuated total reflection (ATR) mode. As well, an uncommon method for PVDF characterization was used ie High Resolution Magic Angle Spinning Nuclear Magnetic Resonance (HRMAS NMR) spectroscopy. Its quantitative aspect has been already proved in some previous works [12]. XPS experiments were performed and core level C_{1S} spectra were decomposed to get more information concerning the surface chemical composition and coverage densities. Chemical coupling of a fluorescent dye on cycloVEGI allowed us to demonstrate that peptide coupling concerned only the outer part of the device and therefore that our goal ie, peptide savings and preserved polymeric support was achieved. Lastly, immobilized peptide recognition by antibodies has been investigated.

2. Experimental

2.1. Reagents. The following chemicals were purchased from Sigma-Aldrich and used as received : acrylic acid (AA) stabilized with 200 ppm hydroquinone, Mohr's salt [$\text{FeH}_8\text{N}_2\text{O}_8\text{S}_2 \cdot 6\text{H}_2\text{O}$], ethyl-3-(3-dimethylaminopropyl)carbodiimide $\text{C}_8\text{H}_{17}\text{N}_3 \cdot \text{HCl}$ (EDC), potassium *ter*-butoxide $\text{C}_4\text{H}_9\text{OK}$ 95%, sulphuric acid H_2SO_4 , N-hydroxysuccinimide (NHS), acetate buffer (pH 5). Hydrophobic PVDF films (PVDF α Atochem, 25 μm thick) were Soxhlet-extracted in toluene and dried at 50 °C under vacuum. Ion-exchange water was used throughout.

2.2. Peptide synthesis and purification. Three linear peptides and a cyclic one have been synthesized: P(236-245) HO-Thr-Trp-His-Val-Leu-Tyr-Ser-Pro-Asn-Val-H (1 213 g/mol), P(237-245) (1114.2 g/mol), P(242-261) H-Ser-Pro-Asn-Val-Ser-Val-Pro-Ser-Ser-Ser-Ser-Thr-Pro-Leu-Leu-Tyr-Pro-Ser-Leu-Ala-OH (2003.2 g/mol) and cycloVEGI, cyclo(DPhe-Pro-Gln-Ile-Met-Arg-Ile-Lys-Pro-His-Gln-Gly-Gln-His-Ile-Gly-Glu) (1998.0 g/mol) [13].

All Solid Phase Peptide Synthesis were carried out on an Applied Biosystems 431A automated peptide synthesizer using dicyclohexylcarbodiimide (DCC)/N-hydroxybenzotriazole (HOBt) activation. On-resin cyclization was performed as previously described [14]. Reversed phase high pressure liquid chromatography (RP HPLC) was performed on a Shimadzu instrument equipped with a SCL-10AVP system controller, LC8A HPLC pumps and SPD-10 AVP UV-vis detector probing at 214nm, 267nm and 254nm on a SATISFACTION RP18AB 5 μm 250x4.6mm C18 column (C.L.I. Cluzeau). The following solvent systems were used for the elution at a flow rate of 1 or 4 ml/min (for analytical and semi preparative HPLC respectively): (A) 0.1% aqueous trifluoroacetic acid (TFA) and (B) 0.1% TFA in 70% aqueous acetonitrile (ACN). MALDI-TOF mass spectrometry was performed on a Reflex III Bruker apparatus.

2.3. Radiation grafting of PVDF with Acrylic Acid [Scheme 1]. Post-irradiation grafting was accomplished by first irradiating the PVDF films at room temperature under Helium atmosphere using a 2.5 MeV Van de Graaf accelerator. PVDF films were irradiated at 150 kGy (dose-rate: 500kGy/h).

Immediately after irradiation, irradiated PVDF films were stored under nitrogen atmosphere at room temperature. Irradiated PVDF films were cut (~ 20*20 mm²), measured (surface and thickness) weighted and characterized by FTIR spectroscopy before grafting. Inside glass tubes, the cut irradiated PVDF films were immersed at room temperature into a grafting solution of AA. Previous study [15] on AA concentration of grafting solution guided us to choose pure AA solution comprising 0.25 wt% of Mohr's salt as grafting solution in order to fit with surface grafting requirements. Mohr's salt was added to avoid any homopolymerization in the liquid phase. Glass tubes were then deaerated under nitrogen during 15 min before sealing. Sealed tubes were then placed into a thermostated silicon oil bath at 60 °C for 1 hour. Grafted films were firstly washed in fresh water and then Soxhlet-extracted with boiled water overnight. Finally, they were dried under vacuum at 50 °C to constant weight. The grafting yield was determined according to the following equation:

$$Y (\%) = \frac{m_f - m_i}{m_i} \cdot 100$$

where m_f and m_i represent the grafted and initial film weights respectively.

2.4. Covalent coupling of peptides *via* a spacer [Scheme 2].

Two coupling procedures were carried out subsequently. PVDF-g-PAA copolymers films [1] (1.5 cm × 2 cm) with a 10 wt % grafting yield (ca. 16 μmol of AA) synthesized previously were first coupled to mTEG in molar range from 16 to 320 μmol. Reaction was carried out in 50 ml of ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) aqueous solution ([mTEG]/[EDC]=1) under nitrogen atmosphere. For that purpose, the PVDF-g-PAA films were firstly swelled in EDC solution for 15 min before the mTEG was added. Reaction times varied from 30 min to 17 hours at room temperature. The resulted films were then immersed in an alkaline potassium *ter*-butoxide solution (KO-*t*-Bu / H₂O = 1:1 w:w) for 2-48 h at room temperature or in an acidic TFA 95 v % solution for 1h at 70°C. Under alkaline conditions, films were washed with deionised water and put into 0.01N H₂SO₄ for 2h. With reactions performed under acidic conditions, films were just washed with deionised water (80 % yield). Saponification or hydrolysis reactions were nearly quantitative (96.5-98% yield).

PVDF-g-PAA-TEG films of (0.75 cm × 1 cm) were activated using 1.76 mM EDC and 1.78 mM N-hydroxysuccinimide (NHS) in 25ml of deionized water for 4 h at room temperature. Films were washed in water. The NHS activated films were immersed for 48 h in a 25ml solution of 0.7 M acetate buffer (pH 5) containing selected peptides (0.339 mg/ml). A blank experiment was also carried out with non-activated PVDF-g-PAA-TEG films in absence of coupling agents under the same conditions to evaluate peptide adsorption. All PVDF-g-PAA-TEG-peptide films were finally washed twice with water.

2.5. Infrared Spectroscopy Measurements. FTIR spectra of the polymer films were carried out with a Nicolet Magna-IR™ 750 spectrometer equipped with a DTGS detector. Spectra were recorded in transmission mode at the Brewster's angle to eliminate interference fringes. To analyze the first micrometers of the film, spectra were recorded in an Attenuated Total Reflection mode (ATR) using a diamond-crystal with single reflection. Both experiments were carried out cumulating 32 scans at a resolution of 2 cm⁻¹.

2.6. Ultraviolet Spectroscopy: determination of peptide consumption. Quantitative determination of linear and cyclic peptides was performed using a UV-visible spectrometer UNICAM UV 300 from 190 nm to 400 nm in acetate buffer (pH 5). Calibration curves are carried out at 280 nm corresponding to tryptophane absorbance in linear peptides and at 220 nm for cyclic peptide which does not contain any tryptophane moities.

2.7. HR MAS NMR Spectroscopy. All experiments were performed by Olivier Assemat (Bruker Biospin S.A., Wissembourg –France) with an Avance 500 spectrometer (500MHz). Material : HR-MAS dual probe (¹H/¹³C) / 4 mm HR-MAS Rotor (ZrO₂) –no insert-. Deuterated DMF, common solvent of both PVDF and PAA, was used. In all ¹H NMR spectra, the water signal at 3.8 ppm was suppressed.

2.8. X-Ray Photoelectron Spectroscopy. Spectra were recorded on a HP5950A spectrometer using a monochromatic Al K α X-ray source (1486.6 eV). The concentric hemispherical electron energy analyzer was equipped with a multichannel detector operating at a constant energy analyzer mode at electron take-off angle of 51.5°. A flood gun was used to cancel charging-up effects. A pass energy of

50 eV was used for both the survey and core level scans. The resolution was between 0.8-1 eV. Binding energies were determined by reference to the C_{1S} component due to carbon bond only to carbon and hydrogen, set at 285 eV. Linear baseline for background subtraction and Gaussian functions were used for peak fitting. Atomic percentages were determined from peak areas by using Scofield factors [16] (C_{1S}=1, F_{1S}=4.43, O_{1S}=2.93, N_{1S}=1.8, Si_{2P}=0.817).

2.9. Confocal Laser Scanning Microscopy. Image was acquired using a Leica TCS SP scanning confocal microscope, with an argon laser of wavelength $\lambda=488$ nm. A plan-Apochromat x40 dry objective with a numerical aperture (NA) of 0.85 was used. Hydrophobic 9 μ m thick β -PVDF film was Soxhlet-extracted in toluene and dried at 50°C under vacuum. Film was irradiated at 100 KGy. Grafting (1h, 60°C) is performed in a pure AA solution with 0.25 wt % of Mohr's salt as described in [2.3]. PVDF-g-PAA film was coupled to cycloVEGI *via* mTEG spacer [2.4]. Last step consisted to covalently link an amine-reactive probe on cycloVEGI using a coupling procedure.

2.10. Peptide/Antibody Recognition.

Peptides functionalized PVDF films were placed inside wells of classical 12-wells plates. The plates were covered and incubated in a blocking solution (3 % BSA in PBS-Tween) under stirring at 37 °C for 1.5 h. After washing in PBS-Tween, 500 μ l of diluted primary antibody DB4 (1/80000 in 1% BSA in PBS-Tween) were added to selected wells. After covering, samples were incubated at 4°C for 18 h under stirring and washed. Then, diluted secondary antibody solution (1/10000 in 1% BSA in PBS-Tween) was added. Plates were covered, incubated under stirring at 37°C for 1 h and washed. TMB (500 μ l/well) was added; plates were covered and incubated at room temperature for 15 min under stirring. Reactions were stopped by 0.5M H₂SO₄. Tests were run in triplicate. Reaction supernatants were transferred to a 96-well plate and absorbance was read at 450 nm in a MRX microplate reader (Dynex technologies). Two controls (one without primary antibody and another without solid support) were run to ensure that the colorimetric reaction was not due to a non-specific reaction.

3. Results and Discussion

3.1. PVDF-g-PAA-TEG-peptide synthesis

Under our experimental conditions, the 2.5MeV accelerated electrons penetrate completely the 9 μ m thick PVDF films. As lineic transfer energy depends on incident electron energy, using less energetic electrons would lead to activate only one side of a 1 mm thick plastic as it is the case for immunoassay plates. Nevertheless, the electron-matter interaction is similar in both cases. Electron beam radiation induces the formation of radical sites in PVDF films. These radicals and some induced peroxides are used as initiators for acrylic acid (AA) polymerization (Scheme 1). Under optimized experimental conditions, surface grafting is favoured [15]. PAA covering is closed to 90%. All these carboxylic acids present onto film surface may bind any molecule having an amine terminal group. In order to confer a biological activity to PAA radiografted PVDF copolymers film [1], peptides will be covalently bound to the grafted films. A first step consists in linking a spacer onto grafted films to bring mobility to the future immobilized peptide. The spacer used, noted mTEG (for modified triethylene glycol), is a functionalized triethyleneglycol with an amine function at one end and a *tertbutyl* protected carboxylic acid at the other end. Covalent linkage of mTEG was achieved through chemical coupling of amino group of the spacer to carboxylic groups of PAA chains using a water-soluble carbodiimide (EDC) (Scheme 2). Best molar yields are averagely 26 mol% of spacer arm covalently bound to [1]. A reaction time of 4 hours and a tenfold excess of spacer and EDC were found sufficient to reach it. The number of spacer molecules was evaluated of around 35 000 per 10 nm² at 26 mol% yield from calculation. This high binding density is 400 times the required space for mTEG molecules tightly closed onto a planar surface from molecular modelling calculation. Except the calculation used to optimize molecular geometry does not take into account physical strain imposed by covalent binding and neighbouring effect, the sponge effect of PAA hydrogel and the non-planar PVDF-g-PAA film surface at a submicroscale can explain such a high calculated density. This parameter is to take into account considering further characterizations in the bulk or on the surface of the films.

Subsequently, the deprotection step occurs to recover carboxylic acid terminal groups of spacer (scheme 2). Two ways of deprotection are studied: in alkaline conditions using potassium *ter*-butoxide (tBuOK) and in acidic conditions using trifluoroacetic acid (TFA). Deprotection kinetic is followed by FTIR in ATR mode. Four hours are needed to obtain a total disappearance of the ester vibration using *t*-BuOK compared to one hour to 80 % decrease using TFA. A vivid violet stains the films during tBuOK treatment. Reducing PVDF-g-PAA-TEG films [3] coloration is possible by diluting tBuOK aqueous solutions (Figure 1A). However, deprotection becomes insufficient. A better result without coloration is obtained in acidic conditions using 95 vol% TFA aqueous solution (Figure 1B). The use of TFA allowed us to optimize the protocol by a one hour deprotection step when heating at 70°C. Following spacer deprotection step, a second coupling reaction is performed to bind peptides. Covalent linkage is then achieved through chemical coupling of its amino groups to the carboxyl acid groups of the spacer using EDC and N-hydroxysuccinimide (NHS) (scheme 2). NHS is used to activate the carboxylic acid groups of [3] to obtain the intermediate, PVDF-g-PAA-TEG-NHS [4]. Primary amine groups are well known to react specifically with NHS activated carboxyl acids. Therefore, there is no need to protect the terminal carboxylic acid of the peptide to avoid peptide homopolymerization. Two peptides are used: a linear one, noted P236-245, and a cyclic one, named cycloVEGI [16]. The last one specifically mimics the VEGF affinity on angiogenesis responsible receptors (see *supra*). The amounts of covalently immobilised peptides are firstly evaluated by performing UV spectra of supernatant peptide solution. Molar yields are calculated as below:

$$Yield(mol\%) = \left[\frac{(C_i - C_f)V}{M_{peptide}} \right] \frac{100}{n}$$

where C_i and C_f are the initial and final peptide concentrations in acetate buffer solutions respectively; V , the peptide solution volume; $M_{peptide}$, the molecular mass of peptide and n , the mTEG mole number. C_i and C_f are determined from maximal UV optical density data after extrapolation from a calibration curve. The resulting yield for linear peptides is found equal to 12 mol% in mTEG equivalent from UV results, similar to 10 ± 2.2 mol% found gravimetrically for PVDF-g-PAA-TEG-

P236-245 [5]. It represents 3500 molecules per 10 nm². When a larger peptide is linked to the polymeric support as it is the case for PVDF-g-PAA-TEG-cycloVEGI films [6], a 1.77 mol% yield of cycloVEGI in mTEG equivalent is found from UV detection at 220 nm corresponding to 500 molecules per 10 nm². Obviously, binding densities are governed by their individual three-dimensional space requirements.

3.2. FTIR characterization

PVDF-g-PAA [1] copolymer films chemical functions are determined by FTIR (Figure 2). In a previous work [17], similar spectra have been discussed in detail. All spectra of [1] contain a characteristic absorption band corresponding to the O=C=O stretching vibration at 1710 cm⁻¹. The carboxylic acids involved in this absorption are associated with the COOH groups of the grafted PAA chains. The PVDF CH₂ stretching vibrations give rise to absorption bands at 3025 cm⁻¹ and 2985 cm⁻¹ and are superimposed on a broad absorption band in the 2500-3500 cm⁻¹ region that corresponds to -OH vibrations of PAA. An unexpected peak at 1555 cm⁻¹ is also observed. It is more or less expressed from a sample to another. By immersing films into an acidic H₂SO₄ solution, the 1555 cm⁻¹ peak decrease contributes to the increase of the COOH peak at 1710 cm⁻¹ (figure 3). This indicates clearly that the peak at 1555 cm⁻¹ corresponds to carboxylates due to the asymmetric stretching vibration of CO₂⁻. Therefore, films [1] were washed in an acidic aqueous solution prior to further chemical modifications. ATR spectra of spacer bound films [2] at 4 h reaction time display no carboxylic acid groups (figure 4). Specific IR peaks of amide bond formation at 1644 cm⁻¹ and 1547 cm⁻¹ are present as well as *t*-butyl, CH₂, ester and ether vibrations at 2980 cm⁻¹, 2860/2870 cm⁻¹, 1726 cm⁻¹ and 1100 cm⁻¹ respectively. *t*-BuOK aqueous solution effects selective cleavage of tertbutyl ester protective acid group -scheme 2-. IR/ATR spectra show the disappearance of the ester peak at 1726 cm⁻¹ and *t*-Butyl vibration and asymmetric stretching at 2980 cm⁻¹ and 1370 cm⁻¹ respectively (Figure 5). FTIR spectra recorded using TFA instead of *t*-BuOK display a residual shoulder of ester peak because of less quantitative cleavage. In Figure 7, ester vibration corresponding to NHS activated carboxylic acid group is clearly illustrated at 1732 cm⁻¹. No carboxylic acid peak is observed showing a total consumption of superficial carboxylic

acids. Therefore, immobilization of peptides can slowly occur in acetate buffer (pH 5) for 48 hours inducing the decrease of the previously formed ester peak (Figure 6). The significant decrease of ester vibration at 1732 cm^{-1} and succinic ester N-O vibration at 1205 cm^{-1} show that most of NHS sites on the surface have reacted. The ester residue is due to a lack of accessibility of NHS activated acids to peptide molecules in taking into account steric hindrance considerations.

3.3. ^1H and ^{13}C HRMAS NMR characterization

After irradiation (150 kGy), some radicals on PVDF films may recombine together to form covalent bonds leading to crosslinked films. As a matter of fact, films are not soluble in DMF. Attempts to obtain solution spectra by classical liquid NMR probe were not satisfactory. Solid state spectra, in such a case, generally display a poor resolution. A solution to this dilemma is to swell the films with the DMF and to acquire spectra while spinning at magic angle. Figure 8 illustrates proton and carbon spectra of a highly grafted PVDF film with a grafting yield of 123 wt% swollen in $\text{DMF-}d_7$. A proton-carbon correlation spectrum was also performed. ^1H spectrum displays large peaks at 1.979 ppm and 1.722 ppm corresponding to the CH_2 of PAA. We would expect only one signal at 1.722 ppm. The chemical shift observed for 33% of the PAA CH_2 at 1.979 ppm may be attributed to CH_2 bound directly on a CF/CF_2 of PVDF or on peroxide. At 2.502 ppm, this large peak of the CH of PAA is superimposed by a triplet corresponding to the CH_3 of PVDF chain ends. These chain ends (around 10 mol% of PVDF) are assumed to come from PVDF chain scissions during radiation process. The large multiplet at 3.038 ppm is the CH_2 of PVDF repeat unit signal. From ^{13}C spectrum of the same product, the CH PAA peak is around 41 ppm and a singlet for COOH is located at 176 ppm. The CH_2 signal of PAA is obscured by spectral overlap with one of the two peaks of the DMF at around 35 ppm. Carbon peaks of PVDF are clearly detected at 23 ppm for chain ends, a triplet at 43 ppm for CH_2 and a well-resolved triplet at 120 ppm for CF_2 . From integrated signals, a quantitative approach permits us to confirm our attributions and evaluate the AA/PVDF ratio. In the present example, an AA/PVDF ratio of 108.5 mol% was found to be compared to 109.7 mol% obtained gravimetrically. For less grafted PVDF film sample, we have found

7.23 mol% by HR-MAS NMR while 6 mol% are obtained from gravimetric measurements. That gives us roughly an error of ± 1.25 mol% showing a relatively good correlation. The proton spectrum of [2] obtained by HR-MAS NMR displays two peaks in the methyl region: a fine one at 1.52 ppm and a larger one at 1.45 ppm both for tertbutyl groups (figure 8 A). Spacer arm molecules on top PAA layer are very mobile and give a nice signal in NMR, contrarily to the ones located inside PAA hydrogel. Indeed, an environment rich in hydrogen bonding possibilities could induce a signal enlargement due to a rather low mobility leading to a poorer spectral resolution. The signals of the CH₂ in the ethylene glycol units overlap with the signal of PVDF. By removing the contribution of DMF, EDC and CH₂ corresponding to ethylene glycol units from integration of PVDF large signal and taking into account both methyl signals as spacer arm ones, 1.57 mol% of spacer/PVDF ratio are detected. This value is in very good agreement with the 1.56 mol% expected value from gravimetric result. After cleavage by *t*-BuOK, the tertbutyl fine signal at 1.52 ppm dramatically decreases to almost complete disappearance while the broad one remains untouched (figure 8 B). It is also interesting to notice in figure 8 B, the disappearance of the broad PAA CH₂ signal. This is due to potassium salt formation. All PAA chains are concerned. As a matter of fact, PAA is no longer soluble in DMF and PAA signals are affected. Quantitative results are not reliable in such conditions. The methyl broad peak presence witnesses a lack of accessibility of spacer arm molecules located inside the PAA hydrogel. The final step is the coupling reaction with a peptide leading to PVDF-g-PAA-TEG-peptide films. For PVDF-g-PAA-TEG-P(236-245) [5], quantitative HR MAS NMR yield is calculated as $2(I_{\text{arom}}/11)/(I_{\text{PVDF}}-6 \times I_{\text{DMF}})$, where I_{arom} is the peak volume of the signal of the 11 aromatic peptide protons, I_{PVDF} is the peak volume of CH₂ PVDF signal contributed for two protons and I_{DMF} is the peak volume of DMF CH at 8.2 ppm (figure 9). That gives us 0.15 mol% of PVDF repeat units for immobilised peptide. In this case, the result is underestimated because we have to remove the peak volume of ethylene glycol units of the spacer overlapped by PVDF signal. After subtracting ethylene glycol integration estimated from previous results, the corrected peptide/AA ratio is finally found equal to 0.18 mol%. In comparison, peptide/PVDF ratios are 0.24 mol% by UV method and 0.19 mol% gravimetrically. HR MAS NMR

technique reveals to be a very accurate method to quantify peptide immobilisation onto PVDF-g-PAA films.

3.4. XPS study

Surface functionalization has been followed by XPS to get more information concerning the surface chemical group composition and coverage densities. As calculated from XPS survey spectra (Table 1), there is a significant increase in N content going from TEG to peptide coupling reactions. Further chemical analysis of PVDF surface modification is obtained from the C(1S) core level spectra depicted in Figure 10. Native PVDF C(1S) shows two peaks at 286.6 eV for CH₂ and one at 291.1 eV for CF₂ with 4.5 eV gap between each binding energy. A slight oxidation of 3.2% is detected due to PVDF oxidation during film storage. The O(1S) corresponding signal was decomposed into two Gaussian peaks at 532.8 eV and 534.6 eV for C=O and C-O respectively (Figure 11). These oxidation defaults are mainly carboxylic acid type. After grafting, three further carbon peaks corresponding to PAA groups are observed at 285 eV, 285.7 eV and 289.4 eV for CH₂, CH and COOH respectively. An oxidation peak of around 5 % has to be added to the decomposition in order to fit properly the experimental data. The O(1S) core level spectrum confirms oxidation presence by a 5 % increase of C=O component relatively to C-O signal. This oxidation may come from initial PVDF film storage and also from polydimethylsiloxane contaminant. When grafting in pure AA, the graft is mainly located to the surface with 81% of coverage. The C(1S) core level spectrum of [2] in figure 10 displays an ether peak at 286.6 eV specific of TEG presence. At that stage, oxidation peak is not detected anymore. The amide peak, present in C(1S) core level spectrum of [2] in the decomposition, dramatically increases at 288.4 eV on C(1S) core level spectrum of [5]. C(1S) core level spectrum of [5] is in agreement with previous studies on peptide characterization [18-19]. The carbon peaks may be decomposed into three major components attributed to carbon-carbon and carbon-hydrogen single and double bounds at 285 eV, to carbon singly bound to nitrogen near 286.4 eV and oxygen at 286.7 eV and to carbon making a double bound to with oxygen such as amide at 288.3 eV and carboxyl acid at 289.6 eV. A $\pi-\pi^*$ characteristic shake-up of

aromatic carbons hold by P(236-245) is also detected at 291 eV. In figure 11, O1S core level spectra clearly shift from carboxylic acid binding energy at 532.4 eV to amide binding energy at 531.5 eV and amide contribution is pointed out as filled area in peak decomposition. O/C, N/C and N/O ratios of [5] are in good agreement to the calculated value of a peptide molecule for 10 TEG spacer molecules giving a 10 mol% yield. Because of XPS detected depth, we can conclude that the peptides are mainly located within the first 8 nanometers of film surface. This result is confirmed by CLMS image displayed on Figure 12 proving that peptide coupling is totally localized onto PVDF polymer surface. Consequently, the PVDF bulk is preserved of any functionalization.

3.6. Peptide/Antibody Recognition

In order to evaluate peptide's activity after immobilization, tests with specific antibodies were performed to determine if molecular recognition is still possible after the grafting of the peptide on the functionalized solid support. For this, an antigenic peptide and a control peptide corresponding respectively to the sequences 242-261 and 237-245 of the Gp46 protein from the Human T-cell Lymphotropic Virus (HTLV-1) were synthesized and immobilized on PVDF-g-PAA. Results (Fig. 13) are evaluated by comparing: (i) the ODs of each samples to their respective control (i.e. without antibody DB4) to evaluate non-specific reaction of the secondary antibody, (ii) the ODs resulting from the samples with antigen and (iii) the ODs resulting from the samples with control peptide to determine non-specific reaction of the antibody DB4. Non-specific reaction with BSA from blocking solution was evaluated for measurement correction. OD variation obtained for PVDF-g-PAA-TEG-antigen compared to all the other films and control experiments is sufficiently significant to conclude that antigen-antibody specificity is maintained. An appropriate cleaning using classical buffer for immunoaffinity chromatography allows the reuse of the functionalized PVDF film for further screening tests.

4. Conclusion

In investigated conditions, electron beam irradiation allows us to obtain a layer of roughly 0.5 μm made up of 90% PAA covalently grafted onto the PVDF film surface without using any toxic chemicals.

This radiation grafting process could be applied to not only PVDF films but also any PVDF devices with varying forms. We have shown that the immobilization of peptides onto PVDF-g-PAA films surface *via* a spacer can be easily obtained by coupling reactions in mild conditions. All chemical functionalizations take place mainly on the films surface as shown from XPS and CLMS results. Yields are governed by steric hindrance considerations. Immobilized peptides are located in the first 8 nanometers of films surface. Among several spectroscopic techniques, HR-MAS NMR appears to be sufficiently accurate to quantify properly immobilized peptides onto a polymeric support. A final recognition test validates the concept of reusable screening assay. This result highlights that immobilization of peptides on this type of polymeric support does not affect their conformations. The work is still in progress in our group to screen novel VEGF-A antibodies with this novel screening assay. PVDF-g-PAA-TEG-peptide copolymer films offer a great biomedical interest. By varying peptide sequences and spacer, it may be possible to develop a panel of applications in screening and/or diagnostic test kits.

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Figure caption

Scheme 1. Radiation grafting scheme (surface grafting conditions: [AA]=100%)

Scheme 2. Reaction scheme of PVDF-g-PAA-TEG-peptide synthesis

Figure 1. a. Deprotection/colour assays onto PVDF-g-PAA-TEG [3] with 10 wt% grafting yield; dose=100kGy; AA=100%; mohr' salt=0.25wt%; t = 4.25 h; tBuOK/H₂O: 1. (2:8); 2. (1:9); 3. (0.25:9.75)~0.2N; 4. 2.10⁻³ N; **b.** PVDF-g-PAA-TEG-P236-245 [5] treated with: 5. tBuOK/H₂O (8:2) and 6. trifluoroacetic acid (95%)aq.

Figure 2. FTIR/ATR spectra of electron irradiated (150 KGy) PVDF and PVDF-g-PAA [1] (1h, 60°C, 100 % [AA])

Figure 3. FTIR transmission spectra of PVDF-g-PAA [1] before (...) and after immersion in H₂SO₄ solution – (---) 5 min; (---) 30 min; (—) 45 min.

Figure 4. FTIR /ATR spectra of PVDF-g-PAA film (1h, 60°C, 100% [AA]) before (---) [1] and after (—) TEG binding [2]

Figure 5. FTIR/ATR spectra of PVDF-g-PAA-TEG before (---) [2] and after (—) [3] cleavage with *t*-BuOK.

Figure 6. FTIR/ATR spectra of PVDF-g-PAA-spacer-COO-NHS [4] and PVDF-g-PAA-TEG-Peptide – 2000-900 cm⁻¹ spectral range-

Figure 7 : HR MAS 500 MHz proton (top) and carbon (bottom) spectra of PVDF-g-PAA [1] films swollen in DMF-*d*₇.

Figure 8 : HR MAS 500 MHz proton spectra of PVDF-g-PAA-TEG films swollen in DMF-*d*₇. ; A. before cleavage [2]; B. After cleavage [3]

Figure 9. HR MAS 500 MHz spectrum of PVDF-g-PAA-TEG-P236-245 [5] swollen in DMF-*d*₇.

Figure 10. C1S core level X-ray photoelectron spectra: (Δ) raw data; Base line; — fit; decomposition: PVDF (▽) CH₂, (■) CF₂; (□) C=O oxidation; (○) CH₂ PAA / C aliphatic + aromatic peptide; (▼) CH PAA; (●) COO PAA/ester TEG/acid peptide; (▲) C-N amide/amine peptide; (◇) CO ether /alcohol; (■) NH-C=O amide; (—•—) shake-up peptide.

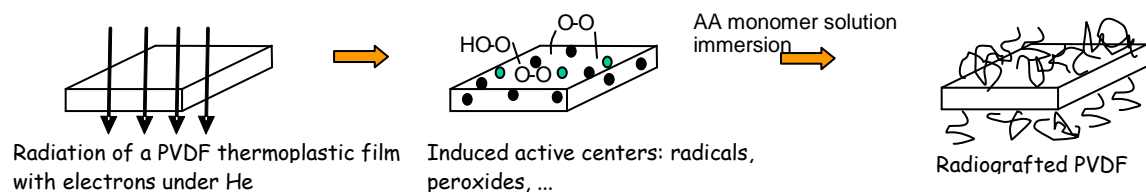
Figure 11. O1S core level X-ray photoelectron spectra: (Δ) raw data; Base line; — fit;(○) C=O; (●) O-C=O; (▲) filled area NH-C=O; (□) C-O-C / C-O-H

Figure 12. Fluorescence image of PVDFβ-g-PAA-mTEG-cycloVEGI film labelled with Oregon Green 488 probes and excited by argon laser Leica TCS SP microscope of 488 nm with a Plan-Apochromat Objective x40 and numerical aperture of 0.85.

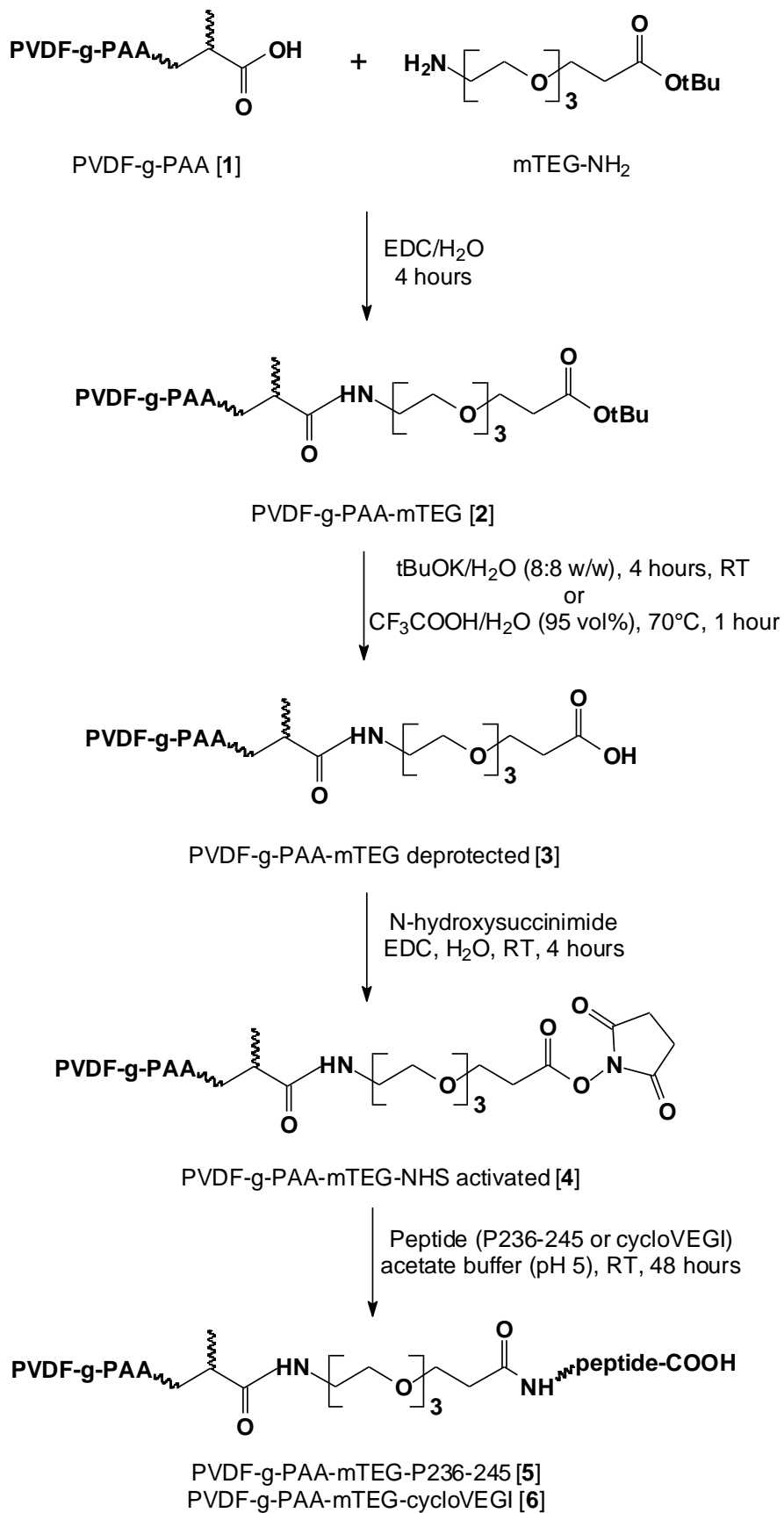
Figure 13. Peptide/antibody recognition test on pristine PVDF, irradiated PVDF, PVDF-g-PAA (1), PVDF-g-PAA-TEG (2), BSA 3% and two PVDF-g-PAA-TEG-peptides (5) containing an antigen P(242–261) and the control P(237–245).

Table caption

Table 1. Atomic percentage of C, O, F, N and Si from XPS for each of the surface functionalization - virgin PVDF, PVDF-g-PAA [1] ([AA]=100 vol %), PVDF-g-PAA-TEG [2] and PVDF-g-PAA-TEG-P236-245 [5]



Scheme 1.



Scheme 2.

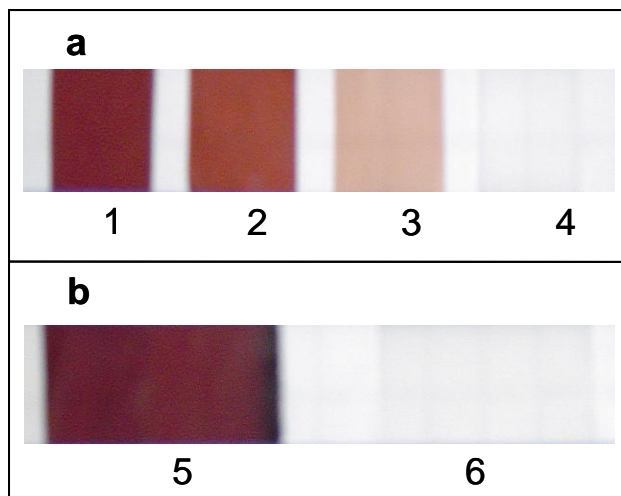


Figure 1.

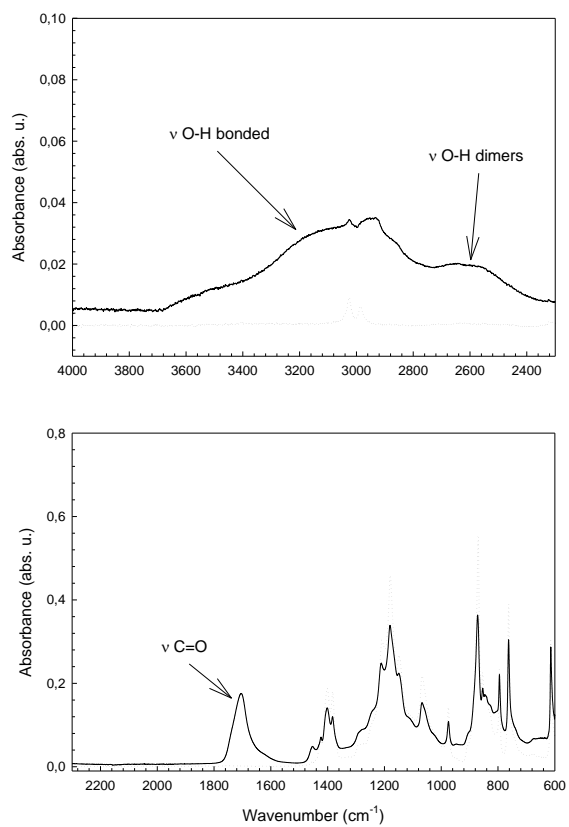


Figure 2.

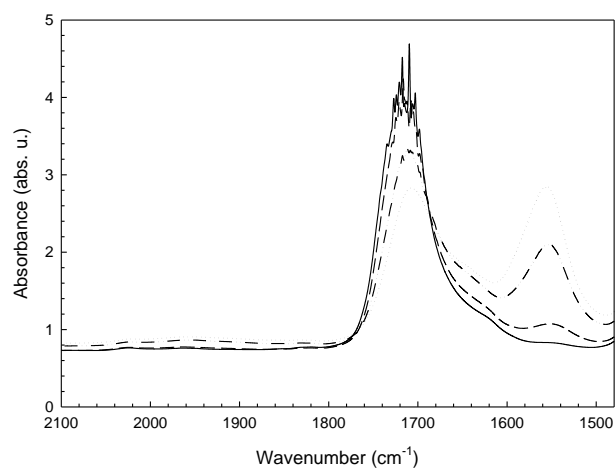


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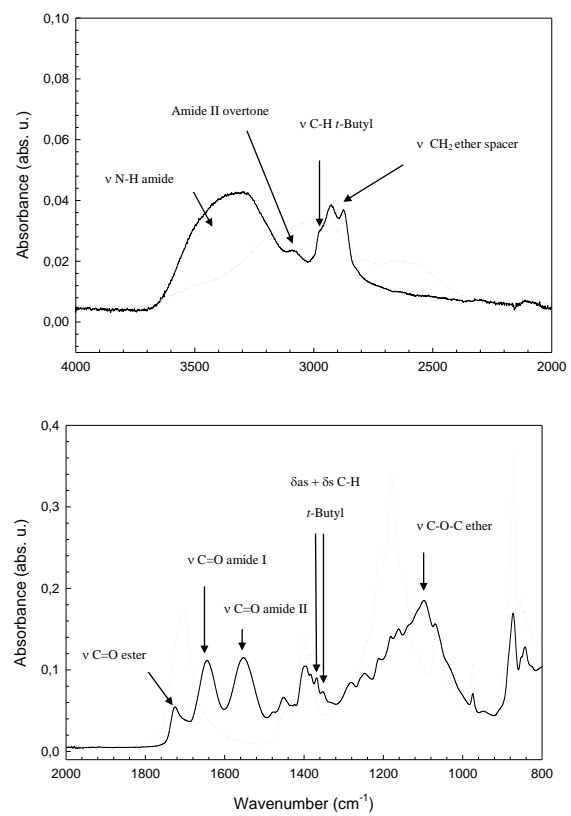


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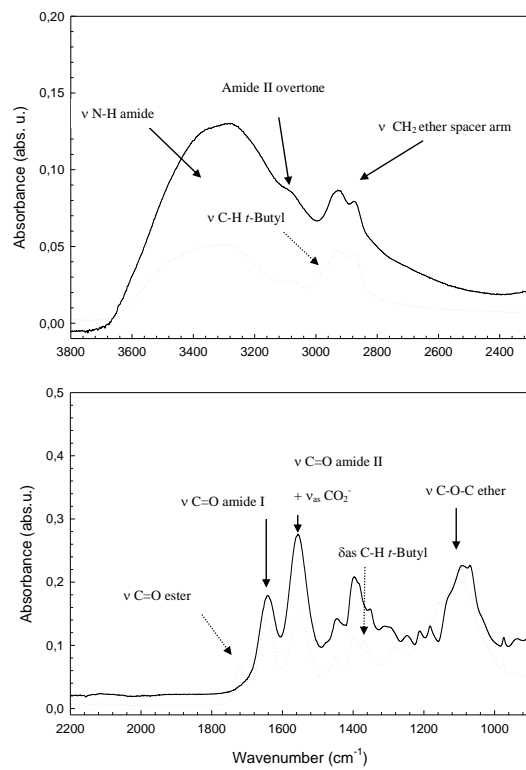


Figure 5.

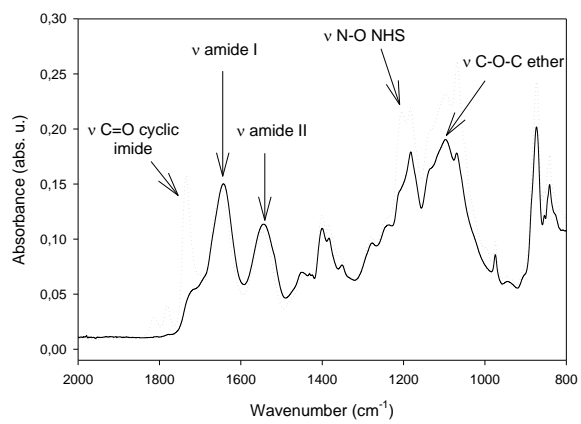


Figure 6.

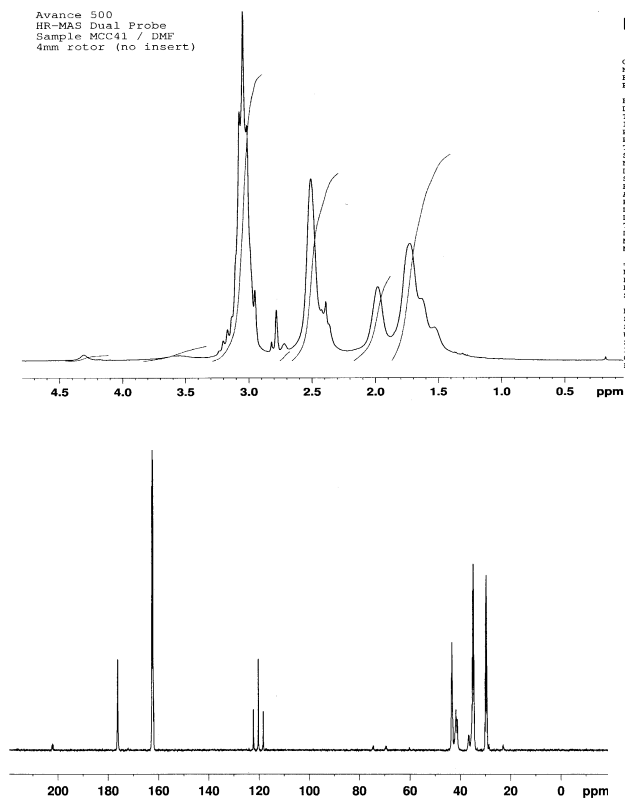


Figure 7.

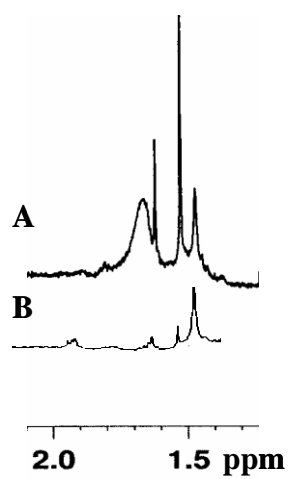


Figure 8.

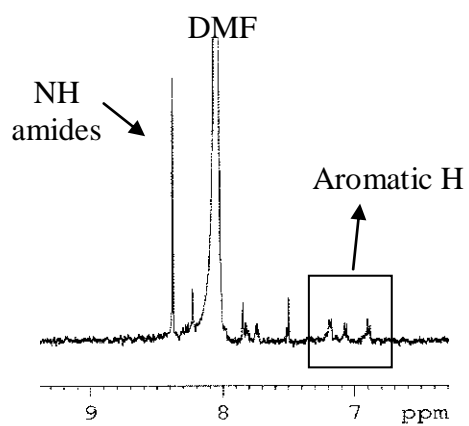


Figure 9.

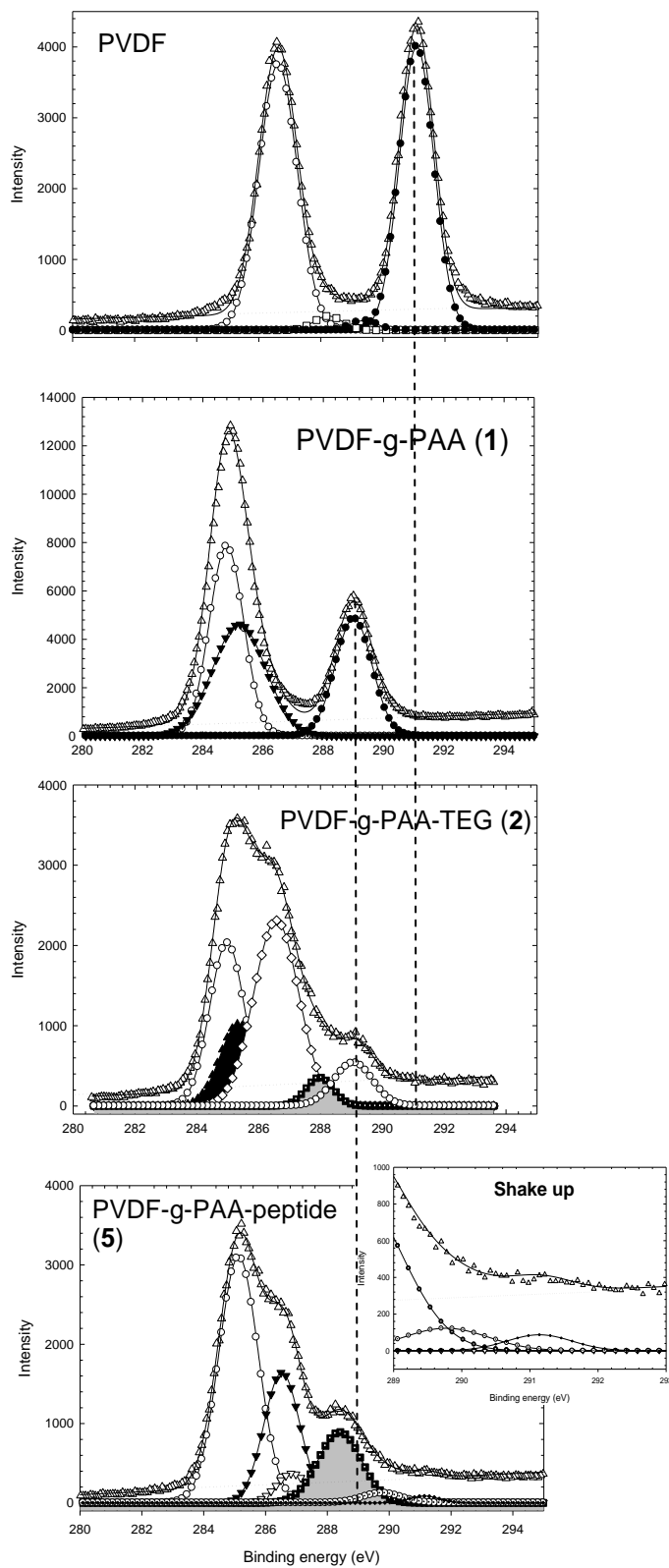


Figure 10: C1S core level X-ray photoelectron spectra: (Δ) raw data; \cdots Base line; $-$ fit; decomposition: PVDF (∇) $\underline{\text{C}}\text{H}_2$, (\blacksquare) $\underline{\text{C}}\text{F}_2$; (\square) $\underline{\text{C}}=\text{O}$ oxidation; (\circ) $\underline{\text{C}}\text{H}_2$ PAA / $\underline{\text{C}}$ aliphatic + aromatic peptide; (\blacktriangle) $\underline{\text{C}}\text{H}$ PAA; (\bullet) COO PAA/ester TEG/acid peptide; (\blacktriangledown) $\underline{\text{C}}-\text{N}$ amide/amine peptide; (\diamond) $\underline{\text{C}}\text{O}$ ether /alcohol; (\blacksquare) $\text{NH}-\underline{\text{C}}=\text{O}$ amide; (\dashrightarrow) shake-up peptide.

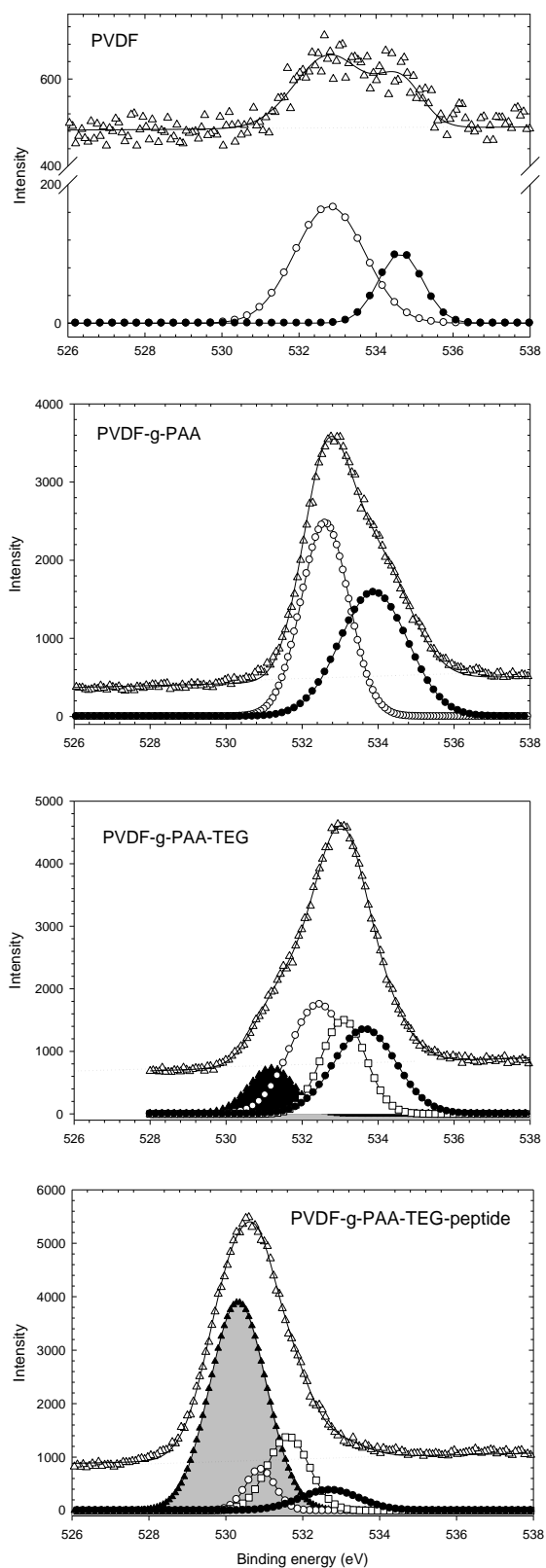


Figure 11: O1S core level X-ray photoelectron spectra: (Δ) raw data; \cdots Base line; $-$ fit; (\circ) C=O; (\bullet) C-O-C; (\blacktriangle) NH-C=O; (\square) C-Q-C / C-Q-H

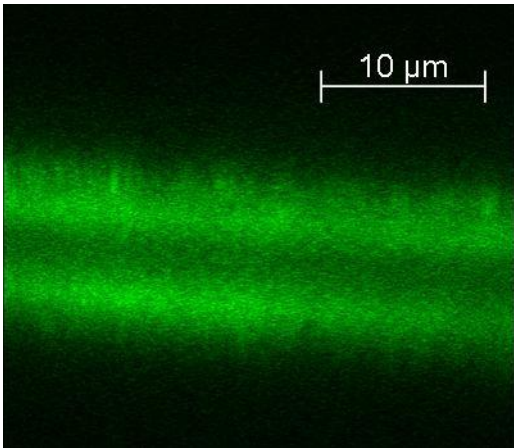


Figure 12.

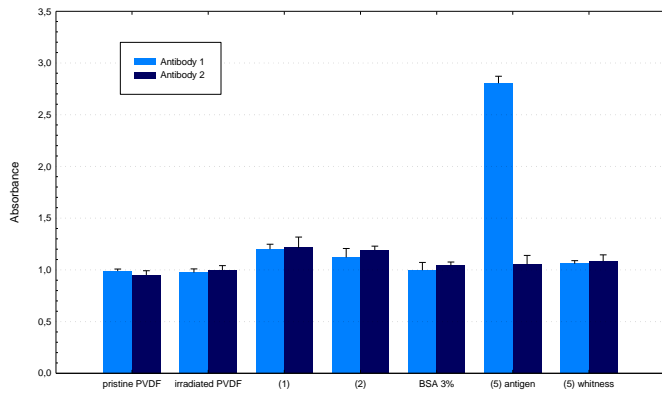


Figure 13.

Table 1. Atomic percentage of C, O, F and N from XPS for each of the surface functionalization - virgin PVDF, surface grafting = PVDF-g-PAA ([AA]=100 vol %), PVDF-g-PAA-TEG [2] and PVDF-g-PAA-TEG-peptide [5]

Samples	C _{1s} (%)	O _{1s} (%)	F _{1s} (%)	N _{1s} (%)
PVDF	49.3	3.2	47.5	0
PVDF-g-PAA	58.63	23.04	3.20	0
PVDF-g-PAA (bis) ^a	66.19	23.45	4.38	0
PVDF-g-PAA-TEG	69.78	21.16	0.24	3.61
PVDF-g-PAA-TEG-peptide	66.25	20.51	0.69	7.96
PVDF-g-PAA-TEG-peptide (bis) ^a	61.25	22.71	0.86	8.78

a. (bis) films which have overcome the same treatment