J Mater Sci: Mater Med (2007) 18:1781–1789
DOI 10.1007/s10856-007-3037-2

A new titanium biofunctionalized interface based on poly (pyrrole-3-acetic acid) coating: proliferation of osteoblast-like cells and future perspectives

Elvira De Giglio · Stefania Cometa · Cosima-Damiana Calvano · Luigia Sabbatini · Pier Giorgio Zambonin · Silvia Colucci · Adriana Di Benedetto · Graziana Colaianni

Received: 22 December 2005 / Accepted: 5 May 2006 / Published online: 5 May 2007

Abstract In recent years, many procedures based on surface modification have been suggested to improve the biocompatibility and biofunctionality of orthopedic titanium-based implants. In this contest, the development of a new titanium-based biomaterial that could be covalently modified with biologically active molecules (i.e., RGD-peptides, growth factors, etc.) able to improve osteoblasts response was investigated. The strategy followed was based on a preliminary coating of the implant material by an adherent thin polymer film to which bioactive molecules could be grafted exploiting the polymer surface chemical reactivity. In this work, we focused our attention on pyrrole-3-acetic acid (Py-3-acetic), a pyrrole with carboxylic acid substituent, whose electrosynthesis and characterization on titanium substrates were already accomplished and whose potentialities in the design of new biocompatible surfaces are well evident. As first step, the biocompatibility of the electrochemically grown PPy-3-acetic films was investigated performing in vitro tests (adhesion and proliferation) with mouse bone marrow cells. Successively, the availability and reactivity of surface carboxylic groups were tested through the grafting of an aminoacidic residue to PPy-3-acetic films.

E. De Giglio (✉) · S. Cometa · C.-D. Calvano · L. Sabbatini · P. G. Zambonin
Dipartimento di Chimica, Università degli Studi di Bari, Via E. Orabona 4, 70126 Bari, Italy
e-mail: e.degiglio@chimica.uniba.it

S. Colucci · A. D. Benedetto · G. Colaianni
Dipartimento di Anatomia Umana ed Istologia, Università degli Studi di Bari, P.zza G. Cesare 11, 70124 Bari, Italy

Introduction

In recent years a great deal of work has pointed out to the development of improved biomaterials, characterized by highly specialized surfaces, able to interact positively with the biological environment. In orthopedic field, particular attention was addressed to the development of bioactive materials that stimulate osteointegration process so to obtain a strong, durable and stable attachment of bone to the implant. In literature, many procedures based on surface modification have been suggested to improve the biocompatibility and biofunctionality of orthopedic titanium-based implants [1–3]. In this contest, our research efforts are addressed to the development of a new titanium-based biomaterial with covalently attached biologically active molecules (i.e., RGD-peptides, growth factors, etc.) able to improve osteoblasts responses. In order to obtain a stable and versatile (''multiresponse'') surface, the implant material was coated by an adherent thin polymer film to which bioactive molecules could be grafted exploiting the polymer surface chemical reactivity. At this aim, there were used conducting polymers which can be electrochemically grown directly onto metallic devices of different shape. Pyrrole and its derivatives yield an important class of electronically conducting polymers that could be promising candidates as implant substrates modifiers due to their interesting biomedical applications [4–8]; moreover, information on polypyrrole biocompatibility with respect to different cell lines [9–13] has been already reported.

In spite of the huge amount of research dedicated to the electrosynthesis of pyrrole in the last two decades, monomers substituted on the 3-position have received much less attention.

This is mainly due to the limited availability of 3-substituted pyrroles, even if this substitution gives the possibility

of introducing specific functional groups on the polypyrrole chain, without interfering with polymerization process.

Pyrroles with carboxylic acid substituents in the 3-position have been employed to produce self-doped polymer films with cation exchange properties and pH sensitivity [14–17], electrode surfaces for direct electrochemistry of cytochrome C [18] and as precursor materials for the preparation of other polypyrrole derivatives [19–21].

In the present work, we focused our attention on pyrrole-3-acetic acid (Py-3-acetic) whose electrosynthesis and characterization on titanium substrates were already accomplished [22]. A spectroscopic investigation performed by X-Ray Photoelectron Spectroscopy (XPS) coupled to a chemical derivatization reaction for COOH groups showed that almost all the monomer units along the PPy-3-acetic chains bear carboxylic functionalities that could be exploited for successive functionalization reactions.

The interface between bone and implant is determined by many factors, including cellular responses to and interactions with the implant. The interfacial events are critical in determining the extent of intimate bone apposition and/or direct chemical bonding of bone and bioactive biomaterials. Therefore, knowledge of the responses of the bone-forming osteoblasts to a solid implant could help in assessing implant performance. To this purpose, in vitro approaches provide ideal systems for studying one cell line at a time without the complications and interferences encountered in vivo when a biomaterial is placed in a bloody wound site in animal models.

Thus, as first step in the development of a titanium-based bioactive material, the biocompatibility of the electrochemically grown PPy-3-acetic film was investigated. In vitro tests with osteoblast-like cells (mouse bone marrow cells) were performed. In particular, cell adhesion and proliferation onto PPy-3 acetic film were evaluated by Titer-tek technique. Successively, the availability and reactivity of surface carboxylic groups were tested through the grafting of an aminoacidic residue to PPy-3-acetic film. The choice of a fluorinated aminoacid such as the 4-fluorophenylalanine allowed the use of XPS analysis to monitor the grafting effectiveness as well as the reaction yield. The results obtained suggest that the surface carboxylic groups could be functionalized, using the same grafting procedure, with a variety of bioactive molecules having amino functionalities, for various bioactive substrates applications.

Materials and methods

Materials and preparations

1(p-tolylsulfonyl)-pyrrole 98% (Aldrich), $Tl(NO_3)_3 \times 3$-H_2O (Aldrich), $AlCl_3$ 99% (Aldrich), CH_2Cl_2 (Aldrich), acetic anhydride $(CH_3CO)_2O$ (Fluka Chemia), $HBFO_4$ (Fluka Chemia), CH_3OH (Fluka Chemia), NaOH (Fluka Chemia), HCl (Fluka Chemia), $LiClO_4$ (Aldrich), pyridine 99.9% (Sigma), CH_3CN 99.93$^+$% (Aldrich), DL-4-Fluorophenylalanine 97% (Aldrich) and 1,3-Dicyclohexylcarbodiimide (DCC) 99% (Aldrich) were used as supplied. Titanium and Ti–Al–V (alloy with 6 at.% Al and 4 at.% V) (Aldrich) sheets (10×10 mm^2), purchased from Aldrich, were mechanically polished by fine diamond paper and then by Al_2O_3 powder (50 μm). After this treatment, before polymer deposition, each electrode was cleaned by an ultrasonic bath using ethanol and successively triply distilled water.

Synthesis of pyrrole-3-acetic acid

Pyrrole-3-acetic acid was synthesized according to a three-step literature procedure [23]. The first step involved acetylation of N-tosyl pyrrole using acetic anhydride in CH_2Cl_2 under Friedel-Crafts condition; in the second step 1-tosylpyrrole-3-methacrylate was obtained via thallation using $Tl(NO_3)_3$. Finally, quantitative hydrolysis of both N-tosyl and methyl ester protecting groups was achieved using 5 M NaOH in methanol at reflux for 12 h. The structure and purity of the resulting pyrrole-3-acetic acid monomer were verified by GC-MS spectroscopy.

Apparatus

All electrochemical experiments were carried out using a PAR 273 potentiostat-galvanostat (EG&G Princeton Applied Research) and a conventional three-electrode system.

In particular, a Pt foil was used as counter electrode and a Ag/Ag^+ electrode (a Ag wire in contact with a 0.1 M solution of $AgNO_3$ in acetonitrile, with a potential +0.35 V vs. SCE) as the reference. The working electrode was a Ti or Ti–Al–V sheet . All the potential values reported in this work were referred to the reference electrode used.

Preparation of poly-pyrrole-3-acetic acid films

Poly-pyrrole-3-acetic acid films (PPy-3-acetic) were electrochemically grown on titanium or TiAlV electrodes in $LiClO_4$ solution (0.7 M in CH_3CN) containing 0.077 g/mL of pyrrole-3-acetic acid. The electrodeposition was performed by cyclic voltammetry in the range $-0.5 \div +0.7$ V.

Solutions were deoxygenated with a stream of N_2 before and during experiments, which were carried out at room temperature. Titanium or TiAlV sheets were mechanically polished before PPy-3-acetic electrodeposition, in order to reduce their native oxide layer and improve the polymer adhesion on their surface, by analogy with PPy

electrosynthesis [5]. Details about electropolymerization process were already reported [22].

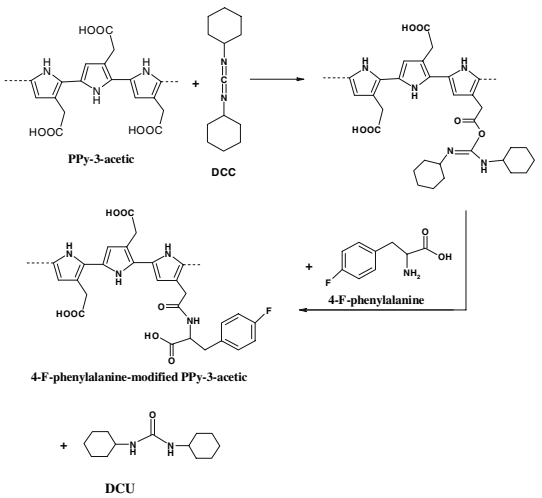
After polymer deposition, the sheets were rinsed with CH_3CN, then with CH_3CN/H_2O 1:1 and finally with triply distilled water.

4-fluoro-phenylalanine-modified PPy-3-acetic films preparation

The 4-fluoro-phenylalanine residues were grafted onto PPy-3-acetic films according to a procedure optimized in order to obtain the highest reaction yield. The optimized procedure was described in the following. Step 1: PPy-3-acetic film was electrochemically grown on Ti or Ti–Al–V sheets. Step 2: PPy-3-acetic-modified titanium substrates were dipped into a solution of DCC (N,N′-Dicyclohexylcarbodiimide), 80 mg in 3 mL of CH_2Cl_2 , for 8 h. Step 3: 0.4 mL of pyridine were added in the same solution. Step 4: After 30′, the sheets were removed from the solution, washed with CH_2Cl_2 and dipped in a solution of 4-fluoro-DL-phenylalanine, 0.05 g in 3 mL of CH_2Cl_2 , for 12 h.

The obtained samples were then rinsed with CH_2Cl_2 , triply distilled water to remove residues of DCU(Dicyclohexylurea) and finally dried by a nitrogen flux before XPS analysis.

The amidic bond formation between aminoacid residues and PPy-3-acetic film was reported in the following scheme:



XPS analysis

XP spectra were obtained using a Leybold LHS10 spectrometer equipped with an unmonochromatized AlK_α X-Ray source and a SPECS electron-analyzer. Wide-scan (kinetic energy range 0–1,500 eV, fixed retarding ratio (FRR) mode with retarding ratio = 30) and high-resolution spectra (fixed analyzer trasmission (FAT) mode, pass energy = 30 eV) were recorded.

Data analysis and curve-fit procedures were performed by means of the SPECSLAB commercial software using the following criteria. The same peak lineshape parameters, Gaussian/Lorentzian ratio and full width at half maximum values were employed for the curve fitting of peaks belonging to the same high-resolution spectrum.

Quantification was performed by using peak areas; comparison between data from different elements was possible after correction (division) by empirically derived atomic sensitivity factors (uncertainty for atomic ratios around 10%).

Cell culture

Ostoblast-like cells (OBs) were obtained from adherent fraction of mouse bone marrow cells [24]. Briefly, hind legs from mice were dissected, the femurs and tibiae excised and soft tissues removed. The bone marrow was harvested by gently flushing the marrow compartment with α-Minimal Essential Medium (α-MEM) (Gibco Ltd, Uxbridge, UK). Nucleated cells were isolated by centrifugation over Histopaque 1077 density gradient (Sigma Chemical Co., St. Louis, MO, USA). After centrifugation, the cells were collected, washed twice with PBS pH 7.4, diluted at 1×10^6cells/mL in α-MEM and supplemented with 10% Fetal Bovine Serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco Limited, Uxbridge, UK) and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. Non-adherent cells were removed and adherent bone marrow cells (BMCs) were cultured and expanded for the experiments. To test the development of osteoblastic cells from undifferentiated BMCs, the culture media described above was switched to osteogenic medium by adding 50 μg/mL ascorbic acid, 0.01 M β-glycerophosphate and 10^{-8} M dexamethasone. Fresh medium was replaced every 3 days. The cells were then trypsinized and transferred to appropriate culture dishes for characterization and experiments. The cells were characterized according to the well established OB parameters such as Alkaline phosphatase (ALP) activity, osteocalcin (OSTC) production and collagen type (COLL I) expression [24].

Alkaline phosphatase assay

Alkaline Phosphatase activity has been evaluated by histochemical and fluorimetrical methods on osteoblasts obtained from mouse bone marrow cells. The cells were cultured in differentiating medium, consisting of αMEM supplemented with 10% FCS, 50 μg/mL ascorbic acid, and 10^{-8} M dexamethasone. Histochemical ALP staining was performed with an appropriate Sigma Kit used on semiconfluent cells. ALP activity on OB lysates was measured by a fluorimetric method, with 4-methyl-umbelliferyl-phosphate used as

substrate. Cell monolayers were solubilized in 0.1% sodium dodecyl-sulphate (SDS). The OB lysates were incubated at pH 10.3 in the presence of 200 μmol/L substrate at 37°C for 30 min. The 4-methyl-umbelliferone (4-MUMBF) produced by the enzyme was detected by monitoring its fluorescence at 369 nm excitation and 448 nm emission wavelengths. The rate of ALP activity was expressed as nmol of 4-MUMBF/min per mg of cell proteins. Protein content was measured by BCA reagent kit (Bio-Rad, Hercules, CA).

RNA isolation and reverse-transcriptase (RT)-PCR amplification

The OBs obtained from mouse bone marrow cells were subjected to mRNA extraction using spin columns (RNeasy, Qiagen, Hilden, Germany), according to the manifacturer's instructions, to detect the expression of ALP, and specific bone proteins secreted by OBs, such as osteocalcin (OSTC) and collagen type I (COLL I). Briefly for the first strand cDNA synthesis (SuperScript First-Strand Syntesis System for RT-PCR, Invitrogen, Carlsbard, CA, USA), a RT mixture containing 1 μg total RNA, dNTPs, Oligo(dT), RT buffer, $MgCl_2$, DTT, RNaseOUT, SuperScript II RT, DEPC-treated water to final volume 100 μL was prepared, according to the manifacturer's instructions. Two μL of diluted cDNA were transferred into a 50 μL PCR reaction mixture containing dNTPs, $MgCl_2$, primers, autoclaved distilled water, Platinum *Taq* DNA polymerase (Invitrogen). Amplification reactions specific for the cDNAs of ALP, OSTC, COLL I and the housekeeping genes glyceraldehyde phosphate dehydrogenase (GAPDH) were carried out using *taq*/polymerase (Invitrogen). The primers and RT-PCR conditions are reported in Table 1. PCR products were analyzed by 1.5% agarose gel electrophoresis containing 0.01% ethidium bromide, and the resulting bands were detected by a light sensitive CCD video system (BioDocAnalyze, Whatman Biometra).

Cell viability assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. OBs were cultured in 24-well tissue-culture plates onto PPy-3 acetic film and onto coverslips as control. Every 24 h for four days, 50 μL/well of MTT 0.5 mg/mL were added, followed by 4-h incubation at 37°C in a humidified 5% CO_2 atmosphere. The reaction was stopped by the addition of 750 μL of 0.04 N HCl in absolute isopropanol. The optical density was read at 570 nm using an automatic plate reader (550 Microplate Reader Bio-Rad Laboratories Inc., CA, USA). The results were normalized to cells incubated under control conditions.

Adhesion and Proliferation assays

OBs were plated onto PPy-3 acetic film at density of 15,000/cm^2 in α-MEM containing 10% FCS. In parallel, OBs plated at the same density onto coverslips were utilized as control. The OB adhesion and proliferation onto PPy-3 acetic film were investigated by Titer-tek technique. Cell adhesion was evaluated after 24 h while cell proliferation was assessed for four days every 24 h. The cells were fixed with 20% methanol for 10 min at room temperature, followed by rinsing with PBS, air-dried and stained with 0.5% crystal violet for 15 min and extensively rinsed. The dye was released from the cells by the addition of 0.1 M Na-citrate in 50% ethanol. The optical density of the released stain solution was read in a Titertek colorimeter (Microtech Bio-rad, Hercules, California) at the wavelength of 540 nm. Results represented the averages of three experiments and were expressed as absorbance ± SE of each treatment performed in quadruplicate.

Statistical analysis

The distribution of the parameters was summarized by reporting mean ± standard error of the mean. Statistical analysis were performed by Student's *t*-test with the Statistical Package for the Social Sciences (spssx/pc) software (SPSS, Chicago, IL, USA). The results were considered statistically significant for $p < 0.05$.

Results and discussion

Cell adhesion and proliferation

Electrically conducting polymers provide potentially interesting surfaces for cell culture in that their properties

Table 1 Primer sequences, annealing temperature, and cycle numbers

Gene	Sense primer	Antisense primer	Annealing Temp.(°C)	Cycle	Product size (bp)
ALP	*5′-GCCCTCTCCAAGACATATA-3′*	*5′-CCATGATCACGTCGATATCC-3′*	55	30	372
OSTC	*5′-AAGCAGGAGGGCAATAAGGT-3′*	*5′-AGCTGCTGTGACATCCATAC-3′*	60	35	292
COLL 1	*5′-GCAATCGGGATCAGTACGAA-3′*	*5′-CTTTCACGCCTTTGAAGCCA-3′*	57.3	30	484
GAPDH	*5′-CACCATGGAGAAGGCCGGGG-3′*	*5′-GACGGACACATTGGGGGTAG-3′*	/	/	418

(e.g., surface charge, wettability and conformational and dimensional changes) can be altered reversibly by either chemical or electrochemical oxidation or reduction. Polypyrrole (PPy) is probably one of the most widely studied conducting polymers due to its chemical and thermal stability, ease of preparation and electroactivity. Therefore, the biocompatibility of PPy becomes an important issue of interest and has been extensively studied in literature in several in vitro systems.

In particular, different authors reported studies performed on PPy with endothelial cells [8], chromaffinoma cell line (PC-12 cells) [9], mesenchymal stem cells [12], nerve tissue cells [13], neuronal cell lines [25] and adrenal chromaffin cells [26, 27]. All the cited studies, together with other in vivo findings [13, 28–30], suggested that this polymer has a good biocompatibility and might be a candidate material for different biomedical applications.

In our previous work, newborne rat calvaria osteoblast adhesion on PPy and RGD-grafted PPy coated titanium substrates was investigated, revealing an increase in cell attachment on RGD-grafted PPy [7]. Moreover, we showed that cell adhesion on unmodified PPy substrates was not significantly different from that on glass coverslips, confirming that this polymer has no toxic effects on osteoblasts.

This finding represented an encouraging starting point for further investigations on PPy-based polymers, such as PPy-3-acetic acid, whose biocompatibility either with respect to osteoblasts or to other cell types has never been tested before.

Therefore, in this study, cell adhesion, growth and viability were evaluated in order to assess the biocompatibility between osteoblast-like cells (OBs) and PPy-3-acetic film.

The OBs obtained from mouse bone marrow (Fig. 1A) were firstly characterized for the well-established osteoblastic parameters. It was observed that ALP activity was strongly evident in the obtained cells (Fig. 1B) and this result was further confirmed by biochemical detection of ALP activity of the same cells (data not shown). Moreover, the mRNA levels of ALP were successively detected in the OB lysates. The use of specific oligonucleotides, as reported in table 1, let us to assess the osteoblastic phenotype of the cells expressing OSTC and COLL I at mRNA levels, indicating that well differentiated OBs were obtained in our cultures.

Since cell adhesion represents an essential process directly involved in cell growth, migration and differentiation, in this study OB adhesion experiments onto PPy-3-acetic film were firstly performed, using coverslips as control. In agreement with evidences obtained in studies [12] on mesenchymal stem cells and in our previous work on rat calvaria osteoblasts [7] seeded onto polypyrrole thin films, here we observed that after 24 h OBs adhered onto PPy-3-acetic film at the same extent of control substrate (data not shown). On the basis of this finding, we consequently evaluated the ability of OBs to proliferate onto the electrosynthesized film. As shown in Fig. 2, OB growth onto the polymer increased with time and did not significantly differ from the control.

In addition, no dedifferentiation state of OB phenotype occurred after adhesion and proliferation experiments, since mRNA levels of ALP, COLL I and OSTC by the cells

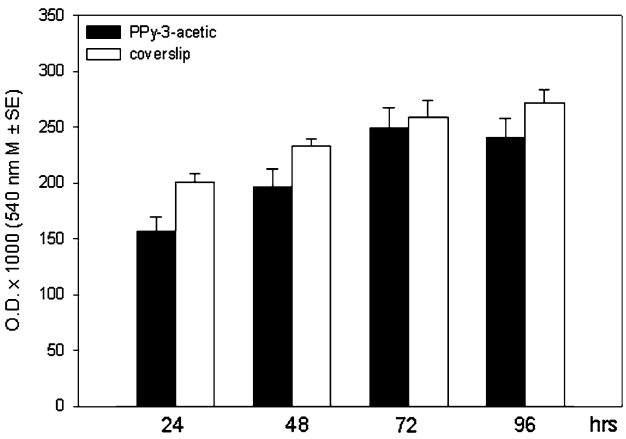


Fig. 2 Osteoblast-like cells proliferation onto PPy-3-acetic film. The growth of OBs plated onto PPy-3 acetic film, evaluated by Titer-tek technique, increases every 24 h as occurs onto coverslips

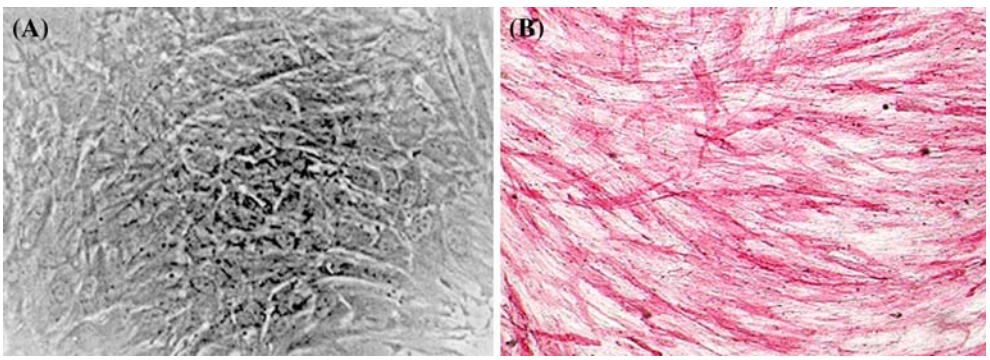


Fig. 1 Osteoblast-like cells generated from mouse bone marrow. OBs, obtained from mouse bone marrow and cultured at confluence (**A**), resulted strongly positive to the histochemical detection of Alkaline Phosphatase (**B**)

were similar to those detected at the beginning of the experiments. These data suggested that PPy-3-acetic represents a suitable substrate to allow not only the adhesion but also the growth of the OBs.

The further step of this study was the evaluation of the OB viability onto the polymer starting from 24 up to 96 h. Mitochondrial dehydrogenases activity has been determined by MTT-test as method to measure cellular activity and proliferation. The advantage of this assay consists in the ability of formazan dye crystals to develop only in living cells, providing an indication of the mitochondrial integrity and activity which, in turn, may be interpreted as a measure of cell viability.

Moreover, the MTT assay performed to assess the cell biocompatibility onto PPy-3-acetic film has never been tested before on osteoblasts, but was only utilized in the case of nerve tissue cells on chemically polymerized PPy [13]. We demonstrated that the tested polymer did not induce any noxious effects either on OB growth or viability and it was comparable to coverslips (Fig. 3). In conclusion, the obtained preliminary results indicated that the well-characterized osteoblast-like cells are able to adhere and proliferate on the electrosynthesized PPy-3-acetic film, and that the cell viability is maintained throughout the time as well as on the coverslip, preserving osteoblastic phenotype. Thus, our results demonstrate that electrosynthesized PPy-3-acetic film shows a good biocompatibility and could be considered a suitable substrate for the bone forming cells.

XPS analysis of 4-fluorophenylalanine-modified PPy-3-acetic films

In a previous work [22] a detailed surface characterization (performed by XPS and SIMS) of PPy-3-acetic films electrosynthesized on both Ti and TiAlV substrates was reported. It was pointed out that the substrate has no significant influence on the polymer film surface composition. Moreover, the presence of COOH functionalities in PPy-3-acetic films was inferred by the curve-fitting of the C1s XP spectra and confirmed by data arising from chemical-derivatization (CF_3CH_2OH)-XPS. In particular, it was found that almost all the monomer units along the PPy-3-acetic chains bear COOH groups. In this work, the availability and reactivity of these surface carboxylic groups was tested through the grafting of an aminoacidic residue to PPy-3-acetic films, performed according to the optimized procedure described in the experimental section. The choice of a fluorinated aminoacid such as the 4-fluoro-phenylalanine is justified by the fact that XPS analysis can easily monitor the reaction effectiveness and its yield by the evaluation of the F1s signal.

The XP survey scan spectra recorded on a PPy-3-acetic film (a) and on a 4-fluoro-phenylalanine-modified PPy-3-acetic film (b) are reported in Fig. 4. The fluorine signal, present in the latter is a reliable indication that the aminoacid grafting reaction occurred. In Fig. 5 the high

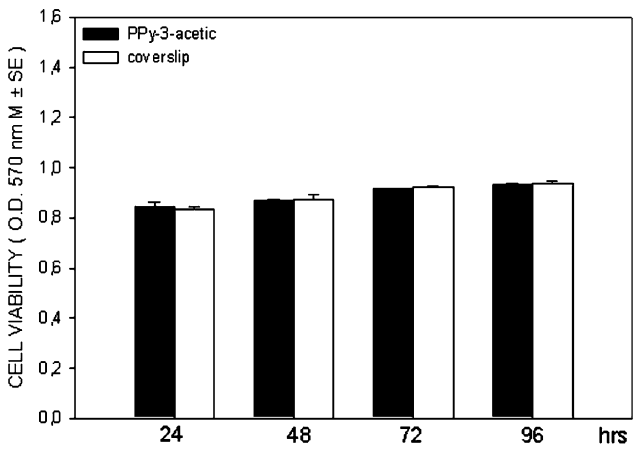


Fig. 3 Osteoblast-like cells viability onto PPy-3 acetic film. Cell viability measured by MTT assay shows the survival of OBs plated onto PPy-3 acetic film similarly to the viability detected onto coverslips

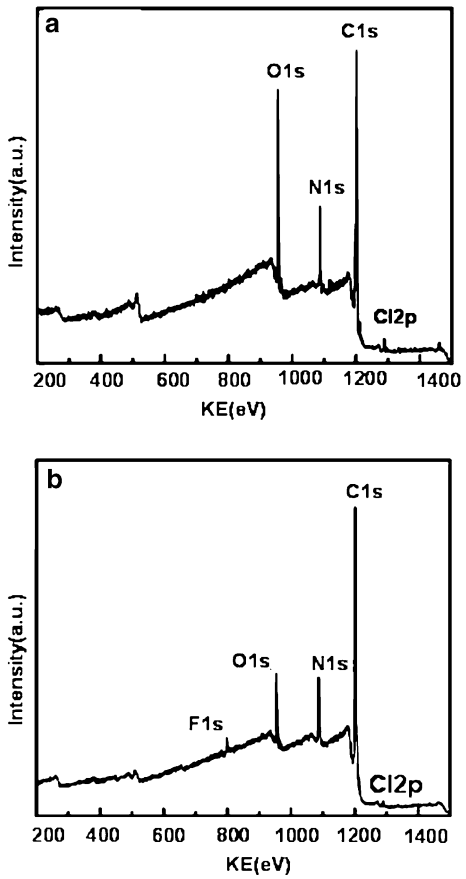


Fig. 4 XP wide-scan spectra recorded on a PPy-3-acetic film before (**a**) and after (**b**) the grafting of the 4-fluoro-phenylalanine

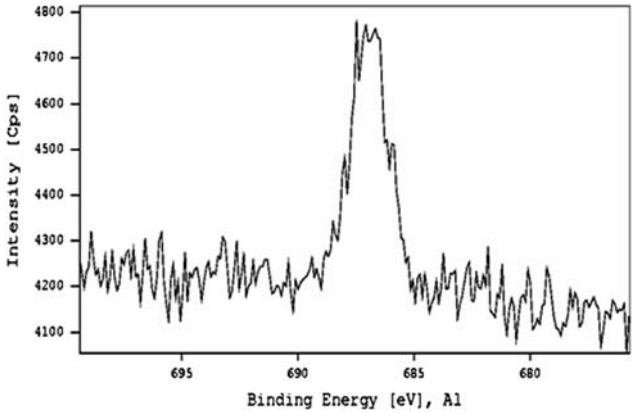


Fig. 5 High-resolution XP spectrum relevant to F1s region for 4-fluoro-phenylalanine modified PPy-3-acetic film

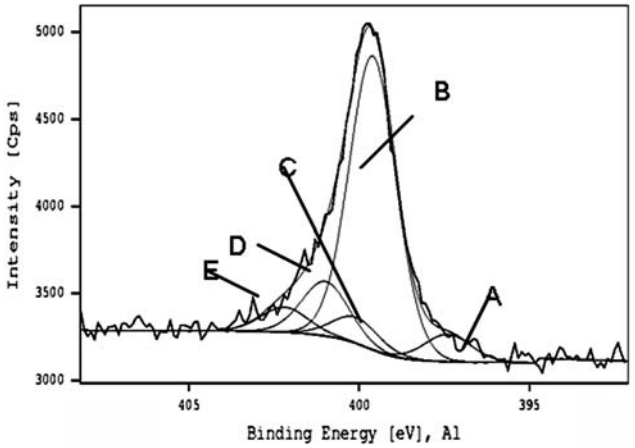
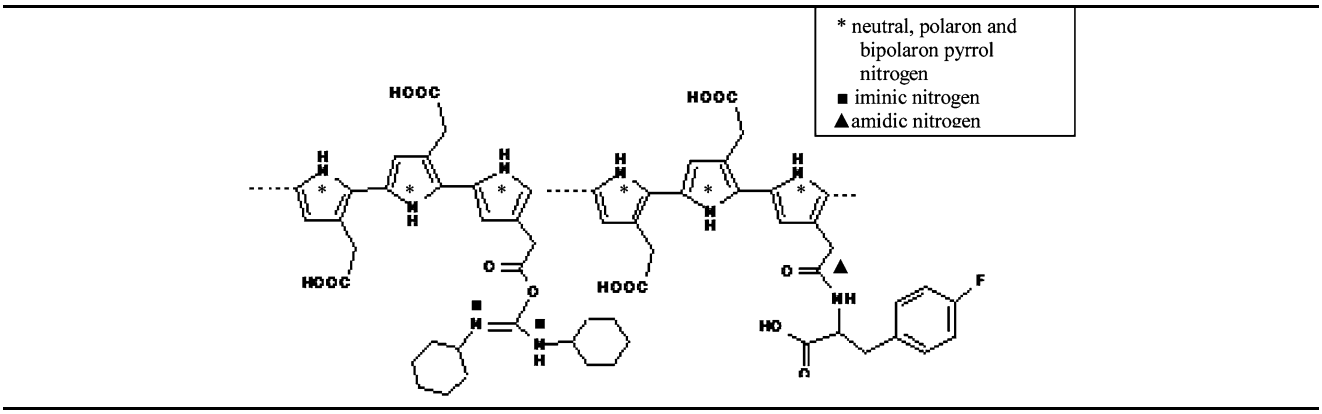


Fig. 6 High-resolution XP spectrum relevant to N1s region for 4-fluoro-phenylalanine modified PPy-3-acetic film. Original data, curve-fitting components and their resultants are also plotted. For peak assignments, see Table 2

resolution F1s spectrum recorded on a 4-fluoro-phenylalanine modified PPy-3-acetic film is shown. In order to estimate the reaction yield, i.e., aminoacid unit/pyrrole unit ratio, the ratio between the high resolution XP F1s signal area (relevant to the aminoacid) and the portion of N1s signal area ascribable to pyrrolic rings was evaluated. At the aim to evaluate the PPy-3-acetic contribute to the N1s signal a detailed fitting of the latter was performed and reported in Fig. 6. Data relevant to binding energies, peak area percentages and assignments of the N1s components for 4-fluoro-phenylalanine modified PPy-3-acetic film are reported in Table 2 together with data obtained on unmodified PPy-3-acetic film on Ti previously reported [22]. N1s fitting components B, D and E, present both in modified and unmodified polymers are ascribable to pyrrole neutral nitrogen, polaron and bipolaron sites respectively. On the other hand, an iminic nitrogen component (peak A), and an amidic nitrogen component (peak C) were detected in 4-fluoro-phenylalanine modified PPy-3-acetic samples. The iminic contribute could be ascribable to DCC molecules still bonded to the polymer, while the amidic component was ascribable to the successful aminoacid grafting, as reported in the following scheme.



It was estimated that the nitrogen peak C area was equivalent, as expected, to fluorine area.

As a result the grafting percentage was evaluated by the ratio between F1s (or nitrogen peak C area) and PPy-3-acetic N1s area (sum of peaks B, D and E), after correction of both the data for their relevant sensitivity factors. This value was found to be equal to 0.08 ± 0.01 (averaged over four samples). This means that one aminoacid over 12 pyrrolic rings was present on the surface of the modified polymer. The obtained value represents a good result in the expectation of developing bioactive substrates through the grafting of biologically active peptides or proteins to titanium implants; nevertheless higher aminoacid concentrations as well as longer

Table 2 Results of XPS qualitative and quantitative analysis (mean ± SD, $n = 4$) for N1s spectra of 4-fluorophenylalanyne modified and unmodified PPy-3-acetic films electrosynthesized on titanium

	4-fluorophenylalanine modified PPy-3-acetic			PPy-3-acetic	
	Assignment	BE (eV)	% Area	Assignment	BE (eV)
$N1s_A$	C=N	397.40 ± 0.05	6.4	–	–
$N1s_B$	N–H (pyrrole)	399.6 ± 0.1	68.9	N-H (pyrrole)	399.6 ± 0.1
$N1s_C$	N–C=O	400.1 ± 0.02	6.1	–	–
$N1s_D$	N^+ (polaron)	400.9 ± 0.2	12.8	N^+ (polaron)	400.7 ± 0.2
$N1s_E$	N^+(bipolaron)	402.2 ± 0.1	5.9	N^+(bipolaron)	402.25 ± 0.15

reaction time could be conveniently used to improve the reaction yield.

Conclusions

In this work, film cell adhesion, growth and viability, in order to verify the biocompatibility between osteoblast-like cells (OBs) and PPy-3-acetic, were evaluated.

The results of our studies indicate that adhesion and proliferation of osteoblast-like cells onto PPy-3-acetic-modified titanium substrates were comparable to those observed in control conditions. Further experiments are currently under investigation to better clarify the osteogenic properties of these cells.

On the other hand, the availability and reactivity of surface carboxylic groups were tested through the grafting of an aminoacidic residue to PPy-3-acetic film. The success of this reaction suggests that the presence of –COOH groups on this polymer film, adherently grown onto implant devices surface, could be conveniently exploited to graft biomolecules (peptides, growth factors, etc.) able to promote positive interface reactions (bioactivity) with the surrounding biological system.

In conclusion, this polymer might be a candidate material for the development of bioactive interfaces in orthopedic field applications.

Acknowledgements We wish to acknowledge the financial support provided by the Ministero per l'Università e la Ricerca (MIUR) and Università degli Studi di Bari. We also like to thank Mr. S. Giacummo for the technical assistance, Mr. A. Tambone (Centro Interdipartimentale di Servizi nel Settore della Spettroscopia) for the technical support in the XPS measurements and Dr. Angela Punzi for her precious collaboration in the chemical synthesis of pyrrole-3-acetic acid.

References

1. M. BROWNE and P. J. GREGSON, *Biomaterials* **21** (2000) 385
2. R. KUMAZAWA, F. WATARI, N. TAKASHI, Y. TANIMURA, M. UO and Y. TOTSUKA, *Biomaterials* **23** (2002) 37
3. E. DE GIGLIO, L. DE GENNARO, L. SABBATINI and G. ZAMBONIN, *J. Biomater. Sci. Polymer Edn.* **12** (2001) 63
4. K. IDLA, O. INGANAS and M. STRANDBERG, *Electrochim. Acta* **45** (2000) 2121
5. E. DE GIGLIO, M. R. GUASCITO, L. SABBATINI and G. ZAMBONIN, *Biomaterials* **22** (2001) 2609
6. E. DE GIGLIO, L. SABBATINI and P. G. ZAMBONIN, *J. Biomater. Sci. Polymer Edn.* **10** (1999) 845
7. E. DE GIGLIO, L. SABBATINI, S. COLUCCI and G. ZAMBONIN, *J. Biomater. Sci. Polymer Edn.* **11** (2000) 1073
8. J. Y. WONG, R. LANGER and D. E. INGBER, *Proc. Natl. Acad. Sci. USA* **91** (1994) 3201
9. C. E. SCHMIDT, V. R. SHASTRI, J. P. VACANTI and R. LANGER, *Proc. Natl. Acad. Sci. USA* **94** (1997) 8948
10. D. TESSIER, L. H. DAO, Z. ZHANG, M. W. KING and R. GUIDON, *J. Biomater. Sci. Polymer Edn.* **11** (2000) 1083
11. E. DE GIGLIO In PhD Thesis National Library, Florence, (Italy), 1997
12. H. CASTANO, E. A. O'REAR, P. S. McFRETRIDGE and V. I. SIKAVITSAS, *Macromol. Biosci.* **4** (2004) 785
13. X. WANG, X. GU, C. YUAN, S. CHEN, P. ZHANG, T. ZHANG, J. YAO, F. CHEN and G. CHEN, *J. Biomed. Mater. Res.* **68 A** (2004) 411
14. P. G. PICKUP, *J. Electroanal. Chem.* **225**(1) (1987) 273
15. T. INAGAKI, M. HUNTER, X. Q. YANG, T. A. SKOTHEIM, H. S. LEE and Y. OKAMOTO, *Mol. Cryst. Liq. Cryst. Sci. Technol. Sect. A* **160** (1988) 79
16. D. DELABOUGLISE and F. GARNIER, *New J. Chem. Technol. Sect. A* **15** (1991) 233
17. P. N. BARTLETT, M. C GROSSEL and E. MILLÁN BARRIOS, *J. Electroanal. Chem.* **487** (2000) 142
18. K. S. RYDER, D. G. MORRIS and J. M. COOPER, *Biosens. Bioelectron.* **12** (1997) 721
19. F. GARNIER, H. KORRI-YOUSSOUFI, P. SRIVASTAVA and A. YASSAR, *J. Am. Chem. Soc.* **116** (1994) 8813
20. B. F. Y. YON-HIN and C. R. LOWE, *J. Electroanal. Chem.* **347** (1994) 167
21. F. GARNIER, H. KORRI-YOUSSOUFI, P. SRIVASTAVA, B. MANDRAND and T. DELAIR, *Synth. Met.* **100** (1999) 89
22. E. DE GIGLIO, C. D. CALVANO, I. LOSITO, L. SABBATINI, P. G. ZAMBONIN, A. TORRISI and A. LICCIARDELLO, *Surf. Interface Anal.* **37** (2005) 580
23. P. GODILLOT, H. KORRI-YOUSSOUFI, P. SRIVASTAVA, A. EL KASSMI and F. GARNIER, *Synth. Met.* **83** (1996) 117
24. M. GRANO, G. MORI, V. MINIELLI, O. BAROU, S. COLUCCI, G. GIANNELLI, C. ALEXANDRE, A. Z. ZALLONE and L. VICO, *Calcif. Tissue Int.* **70** (2002) 176
25. S. LAKARD, G. HERLEM, A. PROPPER, A. KASTNER, G. MICHEL, N. VALLES-VILLARREAL, T. GHARBI and B. FAHYS, *Bioelectrochemistry* **62** (2004) 19

26. T. AOKI, M. TANINO, K. SANUI, N. OGATA and K. KUMAKURA, *Biomaterials* **17** (1997) 1971
27. T. AOKI, M. TANINO, K. SANUI, N. OGATA, K. KUMAKURA, Y. SAKURAI and M. WATANABE, *Synth. Met.* **71** (1995) 2229
28. R. L. WILLIAMS and P. J. DOHERT, *J. Mater. Sci. Mater. Med.* **5** (1994) 429
29. X. CUI, J. WILER, M. DZAMAN, R. A. ALTSCHULER and D. C. MARTIN, *Biomaterials* **24** (2003) 777
30. X. JIANG, Y. MAROIS, A. TRAORE, D. TESSIER, D. TESSIER, L. H. DAO, R. GUIDOIN and Z. ZHANG *Tissue Eng.* **8** (2002) 635