Novel Bioactive Scaffolds with Fibronectin Recognition Nanosites Based on Molecular Imprinting Technology

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ABSTRACT: Biomimetic materials for application in the field of tissue engineering are usually obtained through covalent bonding between the polymer backbone and the bioactive molecules. A totally new approach, proposed for the first time by our research group, for the creation of advanced synthetic support structures for cell adhesion and proliferation is represented by molecular imprinting (MI) technology. In this article, we describe the synthesis and characterization of molecularly imprinted polymers with recognition properties toward a fibronectin peptide sequence and their application as functionalization structures. Polymers, in the form of densely fused microgel particles, were obtained by precipitation polymerization. The imprinted particles showed good performance in terms of recognition capacity and quantitative rebinding; moreover, the epitope effect was observed, with the particles able to recognize and rebind not only the specific peptide sequence but also a larger fibronectin fragment. The cytotoxicity tests showed normal vitality in C2C12 myoblasts cultured in a medium that was put in contact with the imprinted particles. Therefore, imprinted particles were used to functionalize synthetic polymeric films by deposition on their surface. The deposition of the imprinted particles did not alter their specific recognition and rebinding behavior. The most remarkable result was obtained by the biological characterization: in fact, the functionalized materials appeared able to promote cell adhesion and proliferation. These results are very promising and suggest that MI can be used as an innovative functionalization technique to prepare bioactive scaffolds with an effective capacity for improving tissue regeneration. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 3236–3244, 2010

Key words: functionalization of polymers; molecular imprinting; peptides

INTRODUCTION

Biomaterials plays a key role in most tissue engineering strategies. The development of biomaterials for tissue engineering applications has recently focused on the design of biomimetic materials that are capable of eliciting specific cellular responses and directing new tissue formation mediated by biomolecular recognition.[1] The design of biomimetic materials aims to obtain materials that are able to interact with surrounding tissues by specific interactions, mimicking the interactions among cells and extracellular matrix (ECM) proteins in native tissues. Biomolecular recognition of materials by cells has been achieved by two major design strategies. One approach is to endow biomaterials with bioactivity by the incorporation of soluble bioactive molecules, such as growth factors and plasmid DNA, into biomaterial carriers, so that the bioactive molecule can be released from the material and trigger or modulate new tissue formation.[2] The other approach involves the surface and bulk modification of biomaterials by chemical or physical methods with ECM proteins, as well as short peptide sequences derived from them, that can incur specific interactions with cell receptors.[3]

A totally new approach for the creation of advanced synthetic support structures for cell adhesion and proliferation is represented by molecular imprinting (MI) technology.[4–6] This technology, in fact, permits the introduction into a polymeric material of recognition sites for specific molecular species (templates) through the copolymerization of a monomer with a crosslinking agent in the presence of a template[7,8] or through the dissolution of the preformed polymer in a solution containing the molecule to be recognized.[9] In both cases, the spatial arrangement is maintained by the imprinted

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polymer after the template extraction, thanks to crosslinking and rigidity, and confers a selective memory toward this molecule.

MI technology is a valid alternative to the molecular recognition systems present in biological systems. The macromolecular matrices prepared with this procedure, in fact, can be stable even in critical chemical and physical conditions,[10] can have a life of several years without any apparent reduction in their performance, and can be used repeatedly without any alteration to the memory. The most extensively studied applications of molecularly imprinted polymers (MIPs) include artificial antibodies,[11] the separation of macromolecules by chemical affinity,[12,13] biomimetic sensors,[4,5] and intelligent polymers.[14] Interesting developments in the biomaterial field in general[15,16] and in tissue engineering in particular have been proposed in recent years. To the best of our knowledge, our research group has been the first and, until now, unique, to propose MI as a new nanotechnology for the creation of advanced synthetic support structures for cell adhesion and proliferation.[17] Because MI cannot be used directly on biodegradable polymers, which is a fundamental requirement for most tissue engineering applications, our idea was to prepare biostable imprinted particles that could be used in small quantities to modify degradable scaffolds so that these particles would retain their ability to act as temporary supports for cell growth.

This study represents a first step toward the realization of bioactive scaffolds for applications in tissue engineering, which will operate as smart systems for recognizing specific peptide sequences in ECM proteins, to guide and control cell behavior. The hypothesis is to promote cell–material interactions through MIP nanocavities capable of recognizing and rebinding a peptide sequence of the ECM proteins secreted by cells or contained in the culture medium.

The use of entire proteins as template molecules in MI has often been unsuccessful because of the large molecular dimensions and the flexibility of the chains, which limit the polymer molecular recognition capacity and selectivity. To overcome these limitations, stable short peptide sequences, representative of an accessible fragment of a larger protein of interest (e.g., collagen, fibronectin, laminin, vitronectin) can be used; these are often located in the receptor domains or in other parts directly involved in the molecular recognition process (epitopes). Therefore, if the material can recognize a peptide that represents the exposed part of a protein structure, it will also be able to bind the entire protein.[18] In this way, it is possible to increase the adhesion and growth characteristics of the cells on the MIP-modified scaffold because of the presence of sites complementary and selective toward specific sequences within the extracellular proteins and with which the integrin receptorial domains will interact.

In this article, we report the synthesis and characterization of MIPs capable of recognizing a pentapeptide segment of an exposed part of a fibronectin functional domain and their application as functionalization structures in the development of bioactive scaffolds. The choice of the molecule to be recognized was justified by the facts that fibronectin is the main ECM adhesive glycoprotein and it plays a fundamental role in the cell adhesion, migration, and repair processes.[3]

The pentapeptide H-Gly-Arg-Gly-Asp-Ser-OH (GRGDS) was used as a template molecule. The imprinted polymers were prepared by the polymerization of methacrylic acid (MAA) as the functional monomer in the presence of the template molecule and an appropriate crosslinking agent, pentaerythritol triacrylate (PETRA), to provide a sufficient rigidity to the recognition sites in the polymer structure and enhanced hydrophilicity. We characterized the morphological and physicochemical properties of the obtained polymeric materials. The template molecule was removed from the polymers by solvent extraction, and the performances of the obtained polymers in rebinding the template molecule were verified. Cytotoxicity tests were also performed by seeding mouse skeletal myoblasts in a culture medium previously put in contact, for a fixed time, with the polymer. After complete characterization, MIPs were used to functionalize synthetic polymeric films by deposition on their surface. The morphological and physicochemical properties of the composite materials were studied, and the recognition capacity after deposition were also investigated. *In vitro* cell culture tests with C2C12 myoblasts were carried out to evaluate the biocompatibility of the MIP-modified polymeric films and to verify the ability of this new functionalization method for improving cell adhesion and growth.

EXPERIMENTAL

Materials

Polycaprolactone (PCL; number-average molecular weight = 80,000; Aldrich, St. Louis, MO), a triblock poly(ester ether ester) copolymer obtained by the reaction of preformed poly(ethylene glycol) with ε-caprolactone polycaprolactone-poly(ethylene oxide)-polycaprolactone (PCL–POE–PCL)[19,20] and a degradable segmented poly(ether ester urethane) (PU), obtained with poly(ε-caprolactone) diol as the soft segment, an L-lysine–derived diisocyanate and a cyclic diol (1,4-cyclohexane dimethanol) as chain extenders,[21] both synthesized in our laboratory, was used as the substrate to be modified. MAA (>99%), from Aldrich, was purified by distillation *in vacuo* to remove the

polymerization inhibitor. PETRA (Aldrich), azobisisobutyronitrile (>98%, Fluka, St. Louis, MO), and the peptides GRGDS (>97.4%) and H-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-OH (RGDSPASSKP, both from Bachem, Bubendorf, Switzerland) were used as supplied. Acetonitrile (ACN; >99.9%), bidistilled water, methanol (MeOH; >99.9%), and acetic acid (AA) glacial (>99.9%, Carlo Erba Reagenti, Milan, Italy) were of high-performance liquid chromatography (HPLC) grade purity. Trifluoroacetic acid (>99.9%) was from Aldrich. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamine, penicillin, and streptomycin were from Cambrex, East Rutherford, NJ. Paraformaldehyde (PFA), phosphate-buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, and 4′,6-diamidino-2-phenylindole (DAPI) were from Sigma, St. Louis, MO.

Preparation of the MIPs

We prepared the MIPs with a precipitation polymerization method starting from a dilute reacting solution of monomer. The reactors were borosilicate glass tubes sealed with screw caps. The template molecule (0.04 mmol) was dissolved in the polymerization solution, which consisted of an ACN/bidistilled water mixture 70/30 (v/v). Then, the functional monomer MAA (1.18 mmol), the crosslinker PETRA (5.215 mmol), and the initiator azobisisobutyronitrile (0.043 mmol) were added. In the end, the polymerization solution was added to the final volume of 17.5 mL, and the tubes were sealed under dry nitrogen. The polymerization was thermally initiated at 60°C and carried out for 20 h. Constant agitation of the tubes was provided during the entire process. Polymer containing the template in the form of a macroporous monolith was obtained. The macroporous monolith was crushed and washed with ACN to remove the residual monomer and crosslinker and the unbound peptide. We extracted the template by washing the polymers in a Soxhlet apparatus, Franceschi s.a.s., Pisa, Italy for 10 h with an MeOH/AA mixture 90/10 (v/v), and the MIPs were obtained. Nonimprinted (control) particles (NIPs) were prepared with the same procedure of the imprinted polymers, but in the absence of the template molecule.

Preparation of the MIP-modified polymeric films

PCL, PCL–POE–PCL, and PU 2% (w/v) chloroform solutions were prepared and cast on Petri dishes by solvent evaporation at room temperature to obtain homogeneous films.

The polymeric film functionalization was performed through the deposition of imprinted particles on their surface. To allow the adhesion of imprinted particles on the polymeric film surfaces, acetone was identified as a nonsolvent for the particles and as a weak solvent for the polymeric materials used as substrates. Therefore, imprinted particles were suspended in acetone and deposited on the film surface; after acetone evaporation, the film–particle system was lightly washed with distilled water to remove particles that were not perfectly attached. The starting quantity of particles suspended in acetone for each deposition was chosen to have, in the case of complete adhesion and homogeneous distribution, a superficial density of 1 nmol of peptide (molecules or cavities)/cm^2, which could be considered sufficient to promote cell–material interactions, as reported in the literature.[22]

Polymeric films on which NIPs were deposited were used as controls.

The deposition was performed on both sides of the polymeric films. The particle deposition on the polymeric films surface was quantified by weight measurement control (in general, >50% of the starting particles adhered on the film surfaces).

Scanning electron microscopy (SEM)

The morphology of the MIPs and MIP-modified polymeric films were studied with SEM (JSM 5600, Joel Ltd., Tokyo, Japan); the average dimensions of the particles were also estimated. The samples were mounted on metal stubs and coated with gold to a thickness of 200–500 Å with a gold splutter.

Differential scanning calorimetry (DSC)

DSC analysis of the MIPs and MIP-modified polymers was performed in the range 0–200°C with a DSC 7 (Perkin Elmer, Waltham, MA) at a rate of 10°C/min under a nitrogen flow. The endothermic peaks were measured with the DSC 7 software.

Thermogravimetric analysis (TGA)

The level of residual solvent (ACN, MeOH, chloroform, and acetone) in the MIP-modified polymeric films was investigated by TGA with a TGA 6 instrument (PerkinElmer), which achieved a measurement sensitivity of 1 μg. Samples of the MIP-modified polymeric films in triplicate (ca. 10 mg) were heated from 30 to 300°C at a rate of 10°C/min. The occurrence of weight loss peaks were observed at temperatures of 57, 61, 65, and 81°C; these temperatures corresponded to the boiling points of acetone, chloroform, MeOH, and ACN, respectively.

Infrared spectroscopy studies

Attenuated total reflectance (ATR)/Fourier transformed infrared (FTIR) spectroscopy analysis

Infrared spectra of the particles, template, and polymeric films were obtained with a PerkinElmer Spectrum One FTIR spectrometer equipped with an ATR objective lens with a penetration depth of less than 1 μm. All spectra were obtained at 4 cm^{-1} and represented the average of 16 scans.

Infrared imaging

Spectral images of the MIP-modified polymeric films were acquired with an infrared imaging system (Spotlight 300, PerkinElmer). The spectral resolution was 4 cm^{-1}. The spatial resolution was 100 × 100 μm. Background scans were obtained from a region of no sample. IR images were acquired with a liquid-nitrogen-cooled mercury cadmium telluride line detector composed of 16 pixel elements. Each absorbance spectrum composing the IR images and resulting from 16 scans was recorded for each pixel in the μATR mode with the Spotlight software. We collected the spectra by touching the ATR objective on the sample and collecting the spectrum generated from the surface layer of the sample. The Spotlight software used for the acquisition was also used to preprocess the spectra. IR spectral images were produced with the absorbance in a given frequency range, 4000–720 cm^{-1}. Spectra contained in the spectral images were analyzed with a compare correlation image. The obtained correlation map indicated the areas of an image where the spectra were most similar to a reference spectrum. The spectrum of MIP in the range 3500–3000 cm^{-1} was used to obtain the distribution of the imprinted particles on the PU, PCL, and PCL–POE–PCL film surfaces.

Chromatographic analysis

The monomer and crosslinker conversion and the amount of template entrapped by the polymer were determined by the measurement of the corresponding residual amount in the polymerization solution by HPLC (200 series HPLC system, PerkinElmer, with an ultraviolet–visible detector). To determine the monomer and crosslinker concentrations, we used an Alltima C18 5u column (Alltech Associates, Deerfield, IL) (250 mm length × 4.5 mm *i.d.*). The mobile phase was 0.085% (w/v) trifluoroacetic acid in ACN (A) and 0.1% (w/v) trifluoroacetic acid in water (B). The elution condition was an isocratic elution for 15 min with a mobile-phase composition of 54% A and 64% B at a flow rate of 1 mL/min. The injection volume was 100 μL. The detector wavelength was set at $\lambda = 215$ nm. To determine the peptide concentrations, we used a HP Prosphere C4 300A 5u column (250 mm length × 4.5 mm *i.d.*). The elution condition was a linear binary gradient at a flow rate of 1 mL/min, and the gradient was from 30% A and 70% B to 60% A and 40% B in 15 min. The injection volume was 100 μL. The detector wavelength was set at $\lambda = 280$ nm.

Recognition experiments

To evaluate the rebinding capacity of the MIPs and MIP-modified polymeric films, 10 mg of MIPs and NIPs and 1 cm^2 of MIP- and NIP-modified polymeric films were put in contact with a solution of the template in water/ACN 30/70 (v/v). For the MIPs and NIPs, 1.5 mL of a 0.1 mg/mL template solution was used, whereas the polymeric films were put in contact with 1 mL of a 0.0125 mg/mL template solution to obtain a similar template-to-cavities ratio. The samples were maintained under constant agitation for 30 min. The supernatant was separated from the polymer by centrifugation for 30 min at 14,000 rpm. The procedure was repeated three times, with the supernatant replaced every time with fresh template solution. The test was made in triplicate, and the average values were reported. The residual peptide concentration in the rebinding solution was determined by HPLC at wavelength of 280 nm, and the quantity of rebound template was determined by difference. To test the epitope imprinting of fibronectin on the MIPs and, consequently, the ability of the imprinted particles to bind not only the specific peptide sequence but also a larger fibronectin fragment, the same procedure was applied with a solution of the RGDSPASSKP peptide in water/ACN at the same concentration.

Cytotoxicity test

The MIPs were submitted to a cytotoxicity test with C2C12 line cells (European Collection of Cell Culture, London). The cytotoxicity test was an MTT assay. MIPs were sterilized by immersion in an ethanol/water 70/30 mixture three times, followed by UV irradiation for 30 min. A fixed quantity (10 mg) of MIPs was extracted in PBS for 5 days at 37°C. A suspension of C2C12 cells (ca. 10^5 cells/mL) in DMEM containing 10 vol % FBS was distributed in 24 well plates. The MIP extracts were sterile-filtered and then added to half-wells. Sterile PBS was added to the half-wells as a control. The cell culture was carried out in a humidified atmosphere containing 5% CO_2 at 37°C for 72 h. After 24, 48, and 72 h of incubation, cell viability was measured by MTT assay as follows. MTT solution was aseptically added to the wells in an amount equal to 10 vol % of the culture volume, followed by incubation at 37°C for 4 h. After the incubation period, the resulting formazan precipitate was dissolved by the addition of MTT solvent in an

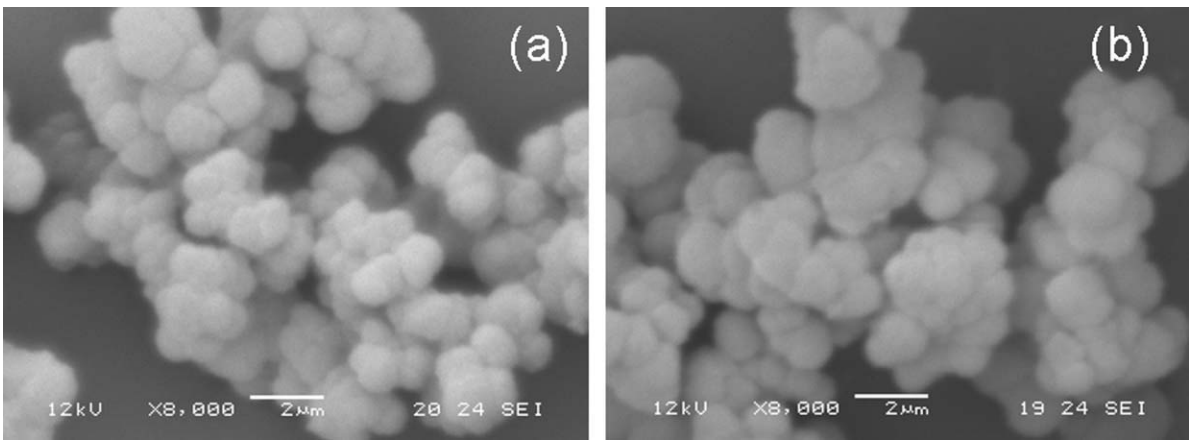

Figure 1 SEM images of the (a) MIPs and (b) NIPs.

amount equal to the original culture volume. The absorbance at 570 nm was measured with a UV spectrophotometer (Shimadzu, Kyoto, Japan).

Cell adhesion and proliferation test

Functionalized polymeric films were prepared for cell culture according to the following procedure. Dry samples, cut into squares of 1 cm^2, were sterilized by washing with 70% (v/v) ethanol solution in sterile water, followed by UV exposure for 15 min on each sample side. Two-dimensional scaffolds were placed in a 24-well plate and seeded with C2C12 (ca. 10^5 cells/mL). Cells were also cultured directly on the wells of the tissue culture plates as a positive control and on blank and NIP-modified polymeric films as a negative control. The growth medium contained high-glucose DMEM, 10 vol % FBS, 2 m*M* glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). The duration of the whole experiment was 7 days; the medium was completely removed every 2 days and replaced with fresh medium.

We evaluated the cell number of the functionalized materials at different times after seeding (1, 3, and 7 days) by labeling cells with DAPI, a fluorescent dye that binds to cell nuclei. At fixed times, the culture medium was removed, and substrates with attached cells were rinsed two times with PBS for 10 min. The attached cells were fixed in 4% (v/v) PFA for 30 min at 4°C and washed more to remove PFA. The fixed cells were subsequently incubated for 2 min in the dark with DAPI and rinsed three times with PBS. Images were collected with a fluorescent microscope.

RESULTS AND DISCUSSION

Physicochemical and morphological characterization of MIPs

The MIPs and NIPs were obtained by precipitation polymerization. After 20 h of reaction, very high monomer and crosslinker conversions were observed (99.5 and 99.95%, respectively) by HPLC analysis of the polymerization solution; this indicated that the reaction went to completion.

The amount of the template molecule contained in the final product, calculated as the difference between the amounts contained in the polymerization solution before and after polymerization, was very high (86.1%). From monomer and crosslinker conversion and residual template in the polymerization mixture, the entrapped template was quantified as 22.4 μmol of GRGDS/g of particles.

The template molecule was removed from the particles by MeOH/AA extraction. The polymer monoliths were crushed and ground in a mortar before this step. The amount of template removed from the particles was not complete (61%) as a consequence of the high crosslinking degree and the rigidity of the matrix. In fact, it has been indicated that bulky templates cannot easily diffuse through a polymer network, even in the presence of a crosslinker containing a polar group that promotes extraction efficiency by increasing wettability. Moreover, this result could have been expected because of the interactions among the peptide and the functional monomer. GRGDS and MAA can create hydrogen bonds, according to two types of interactions: among N—H groups of peptide bonds in GRGDS and C=O groups of MAA and among C=O groups of peptide bonds in GRGDS and O—H groups of MAA.

SEM micrographs showed that the MIPs [Fig. 1(a)] and NIPs [Fig. 1(b)] were composed by densely fused microgel particles. There was no substantial difference in the morphology of the imprinted and control particles in terms of shape and size. All of the samples had a mean diameter of approximately 1 μm.

FTIR spectra of the MIPs (before template extraction), NIPs, and GRGDS showed the following characteristic adsorption peaks of the expected chemical structure: ν(O—H) = 3500/cm, ν(—CH_3,—CH_2) = 2970/cm, ν(C=O) = 1720/cm, and δ(C—O) = 1060/cm for NIPs and ν(N—H) = 3400/cm, ν(N—H,

TABLE I
Rebinding Test for the GRGDS-Imprinted Polymers and Related Controls

	GRGDS		RGDSPASSKP	
	Quantitative binding (μmol/g of polymer)	$\alpha_{MIP/NIP}$[a]	Quantitative binding (μmol/g of polymer)	$\alpha_{MIP/NIP}$[a]
MIPs	7.69	1.34	0.096	1.39
NIPs	5.76		0.069	

[a] Micromoles of peptide bound by MIPs/micromoles of peptide bound by NIPs.

amide I) = 1640/cm, and ν(N—H, amide II) = 1550/cm for GRGDS. The comparison between the MIPs and NIPs did not highlight the presence of the target molecule: the spectra of the MIPs and NIPs did not show any difference in the region among 1700–1500/cm, where the target molecule presented intense adsorption peaks. This result could be explained by the fact that GRGDS was present in the polymerization phase with a concentration below 1.12 wt %. This value was, therefore, below the capacity of the instrument to reveal the peptide entrapped in the polymeric matrix.

The thermal analysis of the MIPs, carried out by DSC, did not show the presence of the glass-transition event because of the high crosslinking degree of the prepared polymers. In the preparation of imprinted particles, a high crosslinking degree is necessary to obtain a rigid structure, which is important for maintaining stable recognition sites in the MIPs produced. As reported in literature, the effect of the crosslinker was to shift the glass-transition temperature toward higher temperatures.[17] The DSC analysis was also performed on the peptide molecule. The thermograms of GRGDS showed several endothermic events above 150°C, which were attributable to the degradation of the peptide. These events were not detectable in MIP thermograms; this could be explained both by the good extraction of the template and by the small amount of peptide contained in the analyzed sample.

Recognition experiments on the MIPs

The recognition properties of the prepared MIPs were investigated by HPLC analysis of the rebinding solution.

The amount of template bound to the imprinted polymer was calculated as follows:

$$\text{Quantitative binding } (\mu \text{ mol of peptide/g of polymer}) = \frac{V(C_i - C_f)}{\text{Grams of polymer}}$$

where V, C_f, and C_i represent the volume of the rebinding solution, the initial solution concentration, and the final solution concentration (concentration after adsorption), respectively.

The imprinting factor ($\alpha_{MIP/NIP}$) was used to evaluate the imprinting effect. It was calculated according to the following equation:

$$\alpha_{MIP/NIP} = \text{Micromoles of peptide bound by MIPs/Micromoles of peptide bound by NIPs}$$

The results of the rebinding test are shown in Table I.

The MIPs were more efficient in template recognition than the related controls; this indicated the formation of specific binding sites in the polymer network. The total amount of template bound was above 7.6 μmol of peptide/g of polymer, a value comparable with those reported for more rigid and smaller templates.[23] It was, however, also evident that the NIPs rebound a significant quantity of peptide because of an unspecific adsorption involving the already mentioned hydrogen-bond interactions among the carboxylic groups of MAA and the polar groups of GRGDS.

Also, the resulting MIPs were able to recognize the fibronectin fragment containing the Arg-Gly-Asp (RGD) sequence with the same $\alpha_{MIP/NIP}$ ($\alpha_{MIP/NIP}$ = 1.39), which confirmed the success of the epitope approach.

Cytotoxicity test

Figure 2 shows the results of the cytotoxicity test, in which MIP extracts were put in contact with C2C12 myoblast cells for 24, 48, and 72 h.

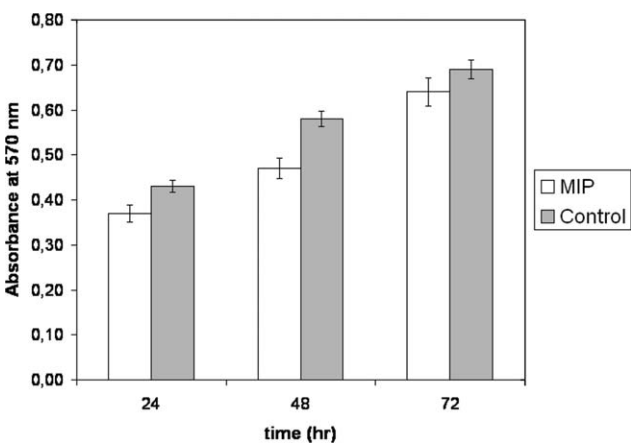

Figure 2 Cytotoxicity test of the MIPs with C2C12 myoblasts cultured in a culture medium conditioned with MIP extracts. Cells cultured in a medium not incubated with imprinted particles were used as controls.

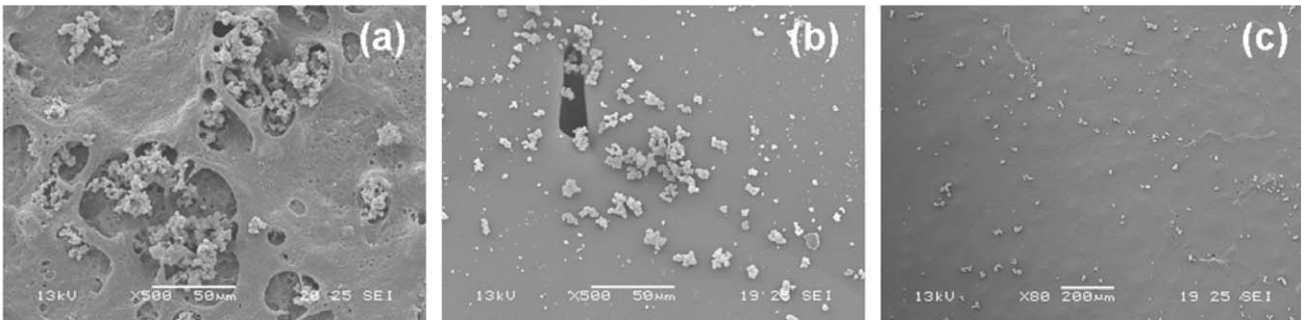

Figure 3 SEM images of the polymeric film surfaces modified by imprinted particle deposition: (a) PU, (b) PCL, and (c) PCL–POE–PCL.

Cell viability, evaluated by MTT assay, was always maintained very close to that of the control. These results show that the imprinted particles had no cytotoxic activity against C2C12 myoblasts.

Physicochemical and morphological characterization of the MIP-modified polymeric films

Figure 3 show the morphology of the polymeric film surfaces after the deposition of the imprinted particles. SEM micrographs confirmed the presence of the particles on the polymeric film surface. A quite homogeneous particle distribution was observed, although the particles tended to form clusters and the MIP density was higher in proximity to small cavities or defects on the polymer surface. In particular, in the case of PU [Fig. 3(a)], the deposition process determined a superficial modification with the formation of circular alveoli that, however, could promote interactions among the cells and the substrate.

The results of the thermal analysis, carried out by DSC, are reported in Table II. Variations in the melting enthalpy for the functionalized materials were observed. This result suggests that the presence of particles altered the crystallinity of the composite materials. The reason could be the partial solubilization of the polymeric film surface, necessary during the functionalization process. It is well known that the degree of crystallinity in cast polymeric films depends on several parameters, such as the casting solvent and solvent evaporation rate.[24]

TGA of MIP-modified polymeric films showed no weight loss at temperatures corresponding to the boiling points of the solvents used; this showed that no trace of solvent was present in the functionalized materials.

Infrared imaging

FTIR spectra relative to the MIPs and polymeric films were acquired. The spectra showed the characteristic adsorption bands of the polymers used. The comparison among the spectra allowed us to determine a frequency range diagnostic of the presence of the MIPs on the polymeric films. This range was identified in the 3500–3000-cm^{-1} region, which corresponded to the OH groups of poly(methacrylic acid), that is, the polymer of which the MIPs were made.

In Figure 4(a–c) are shown, respectively, the chemical map, the correlation map, and the spectra recorded in different regions of the correlation map compared with the reference spectrum of the MIPs for the MIP-modified PU. The presence of the characteristic adsorption band in the range 3500–3000 cm^{-1} in all of the acquired spectra demonstrated a quite homogeneous distribution of the MIP aggregates on the PU film surface. The same analysis was performed for MIP-modified PCL and PCL–POE–PCL, as shown in Figure 4(d–i), respectively. The results show the presence of the characteristic adsorption band, which confirmed good distribution of the imprinted particles on the surfaces of the PCL and PCL–POE–PCL films.

Recognition experiments of MIP-modified polymeric films

The rebinding properties of the MIP- and NIP-modified polymeric films are shown in Table III.

The deposition of the imprinted particles did not alter their specific recognition and rebinding behavior. Moreover, the functionalized films showed a

TABLE II
Melting Enthalpy (ΔH) for the Blank and MIP-Modified Polymeric Films

	ΔH_{PCL} (J/g)[a]	$\Delta H_{PCL-POE-PCL}$ (J/g)[a]	ΔH_{PU} (J/g)[b]
Blank film	73	109	51
MIP-modified film	84	94	53

[a] Melting temperature $T_m = 66°C$.
[b] Melting temperature $T_m = 50°C$.

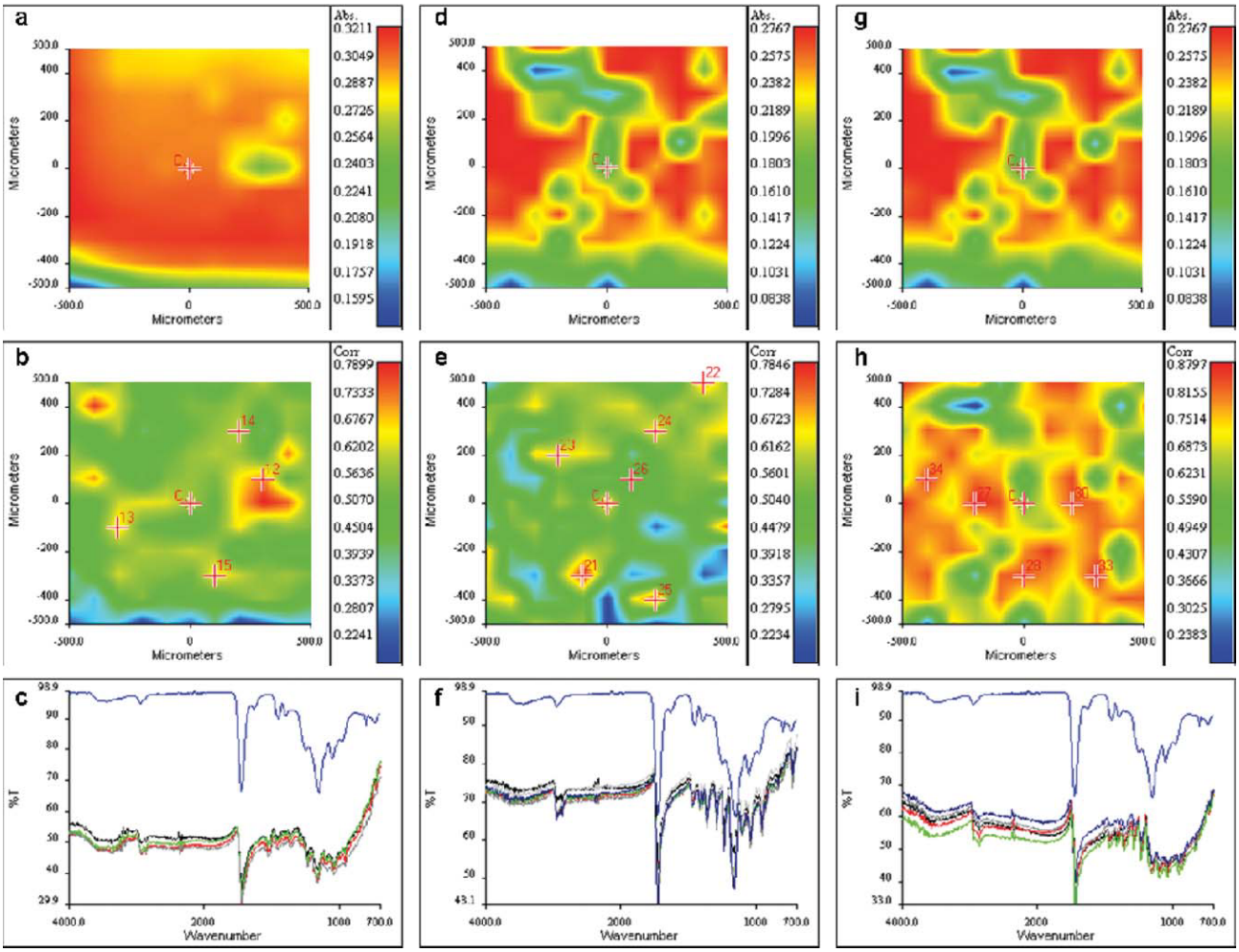

Figure 4 (a,d,g) Chemical maps, (b,e,h) correlation maps, and (c,f,i) FTIR spectra acquired in different regions of the correlation maps for MIP-modified PU, PCL, and PCL–POE–PCL, respectively. The presence of MIPs on the polymeric film surfaces was demonstrated through the characteristic adsorption band in the 3500–3000-cm^{-1} region. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

higher quantitative binding than the free particles (see Table I); this suggested the creation of a preferred microenvironment for the rebinding process. In particular, PU was the most efficient substrate, probably because the deposition produced a superficial modification, which could promote the binding performance.

Cell culture test

We evaluated cell proliferation at fixed times after seeding by counting the number of cells after their labeling with DAPI. The results (Fig. 5) show that the MIP-modified polymeric films significantly increased cell proliferation with respect to blank and NIP-modified materials. In particular, the MIP-modified PU films showed higher values of cell proliferation with respect to functionalized PCL and PCL–POE–PCL; this confirmed the good performance obtained in the rebinding experiment by the same materials. Comparing the behavior of the functionalized materials with the positive control, we observed that although 1 day after seeding, the cell number on the MIP-modified polymeric films was around 75% of that

TABLE III
Rebinding Performance of the Polymeric Films Functionalized with MIPs

	Quantitative binding (μg of GRGDS/cm^2)	$\alpha_{MIP/NIP}$[a]
PCL plus NIPs	16.32	–
PCL plus MIPs	28.88	1.77
PCL–POE–PCL plus NIPs	20.41	–
PCL–POE–PCL plus MIPs	34.04	1.67
PU plus NIPs	19.84	–
PU plus MIPs	36.09	1.82

[a] Micromoles of peptide bound by films functionalized with MIPs/micromoles of peptide bound by films with NIPs.

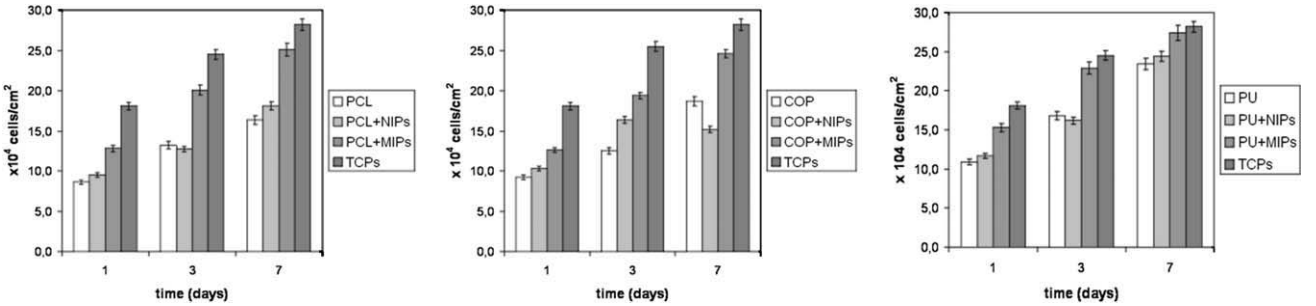

Figure 5 C2C12 myoblast proliferation on the MIP- modified (a) PCL, (b) PCL–POE–PCL, and (c) PU films. Blank and NIP-modified PCL films were used as negative controls; cells cultured directly onto the tissue culture plates were used as positive controls.

obtained on the tissue culture plates, in the following days, these values of proliferation became very close. The reason for this result could be found in the type of cell–material interactions aimed by MI. In contrast with traditional functionalization strategies, in which bioactive molecules are exposed on the scaffold surface and, therefore, cell adhesion is directly promoted, when molecularly imprinted particles are used as functionalization devices, the cell–material interaction are indirectly promoted, and the ECM proteins contained in the culture medium or secreted by cells are recognized and rebound by MIP nanocavities.

CONCLUSIONS

The aim of this study was to develop innovative bioactive materials for tissue engineering applications through MI. MIPs with recognition properties toward the fibronectin GRGDS peptide sequence were synthesized and characterized. The obtained results reveal possible applications for MIPs as recognition materials for cell adhesive proteins; therefore, the functionalization of synthetic polymers was carried out by the deposition of MIPs on their surface. MIP-modified polymeric films underwent a complete morphological, physicochemical, functional, and biological characterization. We demonstrated that the deposition of imprinted particles did not alter their specific recognition and binding behavior, and the quantitative binding was even improved because of the creation of a preferred microenvironment for the rebinding process. The most remarkable result was obtained in the biological characterization: MIP-modified polymeric films significantly increased cell proliferation with respect to nonfunctionalized materials, with rising values very close to that offered by the positive control. These results are very promising and suggest that MI can be used as an innovative functionalization technique to prepare bioactive scaffolds, with an effective capacity for improving tissue regeneration.

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