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Nano-Colloid Printing of Functionalized PLA-*b*-PEO Copolymers: Tailoring the Surface Pattern of Adhesive Motif and its Effect on Cell Attachment

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Summary

In this study, we investigate the preparation of surface pattern of functional groups on poly(lactide) (PLA) surfaces through the controlled deposition of core-shell self-assemblies based on functionalized PLA-*b*-PEO amphiphilic block copolymers from selective solvents. Through grafting RGDS peptide onto the functionalized copolymer surface, the presented approach enables to prepare PLA surfaces with random and clustered spatial distribution of adhesive motifs. The proposed topography of the adhesion motif was proved by atomic force microscopy techniques using biotin-tagged RGDS peptide grafted on the surface and streptavidin-modified gold nanospheres which bind the tagged RGDS peptides as a contrast agent. The cell culture study under static and dynamic conditions with MG63 osteosarcoma cell line showed that the clustered distribution of RGDS peptides provided more efficient initial cell attachment and spreading, and resistance to cell detachment under dynamic culture compared to randomly distributed RGDS motif when with the same average RGDS peptide concentration.

Key words

Tissue engineering • Surface pattern • AFM • Poly(DL-lactide)-poly(ethylene oxide) block copolymer • Cell attachment

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Introduction

The role of polymer biomaterials in tissue regeneration/engineering is to provide a temporary scaffold for the growing tissue. The polymer scaffold represents a synthetic analogue of the native extracellular matrix (ECM). Therefore, besides being a mechanical support, the scaffold must also provide additional cues that mimic the ECM-to-cell signaling mechanisms. To this end, a lot of effort is focused on creation of cell/material interfaces with incorporated adhesive motifs, mostly derived from ECM components such as fibronectin or laminin, to facilitate cell adhesion, migration, growth or differentiation (Goddard and Hotchkiss 2007, Tejeda-Montes *et al.* 2014). For incorporation of biomimetic groups, the surface must be first resistant to the non-specific protein adsorption, otherwise the present biomimetic motifs would be masked by an adsorbed layer of proteins that occurs at the first instant after the biomaterials is brought into contact with a protein-containing biological fluids (Chen *et al.* 2008). In this respect, a surface coating with poly(ethylene oxide), PEO, has proven to be efficient in creation of a non-fouling surface. The biomimetic structures, such as adhesive motif, e.g. RGDS (arginine-

glycine-aspartic acid-serine) peptide derived from fibronectin, attached to otherwise non-adhesive surface can provide specific sites recognizable by integrin cell receptors and facilitate the cell adhesion (Cavalcanti-Adam *et al.* 2006, Goubko *et al.* 2014).

However, not only the presence of biomimetic peptides but also the density and spatial distribution of adhesion peptides have been proved to play a significant role (Massia and Hubbell 1991) in order to cluster cell receptors integrins into focal adhesions, a key structure in signal transmission, as well as in differentiation of human mesenchymal stem cells (Frith *et al*. 2011). The maximum spacing between RGDS-groups on the surface for formation of focal adhesion for several cell types was established (Arnold *et al*. 2004) and the influence of RGDS-spacing on cell adhesion strength has been investigated (Selhuber-Unkel *et al.* 2010).

There are physical techniques of surface modification available for model studies on well-defined substrates, such as flat silica, gold or glass, e.g. lithographic methods. However, working with lateral resolution on a nanometer scale, there are only limited options to form analogous effects on diverse biomaterials, such as biodegradable polymers in their complex 3D shapes of tissue engineering scaffolds. In this work, we approach this problem by taking advantage of self-assembly of amphiphilic block copolymers composed of hydrophobic PLA and hydrophilic PEO blocks in selective solvents and their organized deposition on the material/solution interface, particularly on polyester-based biomaterials such as polylactide. This technique could be potentially applied for 3D systems.

In our previous studies, the efficiency of the polylactide surface modification through a surface deposition of PLA-*b*-PEO amphiphilic block copolymers has been investigated. The formation of a copolymer layer and its efficiency in preventing the non-specific protein adsorption compared to unmodified PLA surfaces was investigated by Popelka *et al.* (2007). Furthermore, Tresohlava *et al.* (2010) demonstrated that some of the PLA-*b*-PEO block copolymer molecules in the layer can carry a bioactive molecule (or functional group in general) bound to the end of the PEO chain which, after the deposition of the copolymers on the surface, remains accessible for a molecularly specific interaction with a protein, despite that a general protein adsorption was suppressed. In the most recent study we demonstrated that using a larger marker, such as modified gold nanospheres with significantly different mechanical properties compared to the origin polymer layer, the individual adhesion peptide molecules grafted to the polymer surface can be labeled and detected by monitoring of local mechanical properties by AFM methods (Knotek *et al.* 2013).

In the present study we investigate the deposition of amphiphilic block copolymers for creation of a nanoscale pattern of the biomimetic RGDS peptide motif and the effect of thus prepared surfaces on initial cell adhesion. The PLA-*b*-PEO copolymers in selective solvents form micelles or, generally, colloidal self-assemblies, typically with a core-shell structure, where the condensed core is composed of the hydrophobic (PLA) blocks, whereas the expanded hydrophilic (PEO) blocks form a hydrated shell of the particle.

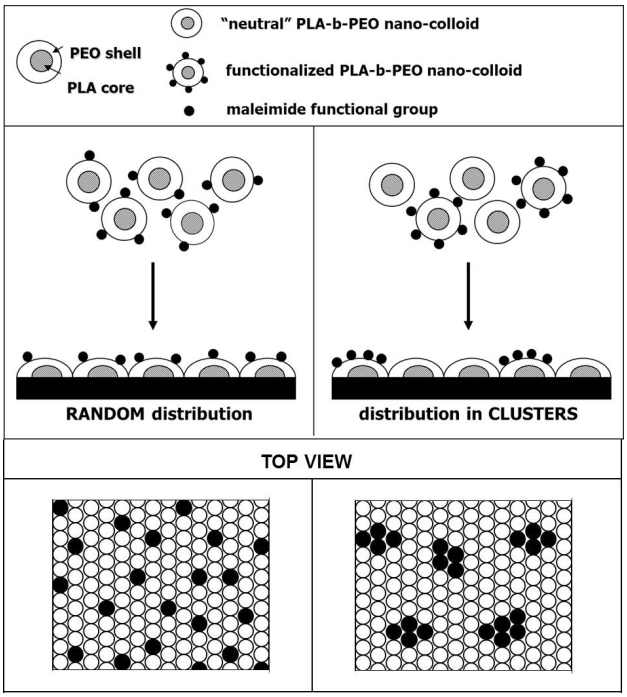

Fig. 1. The schematic drawing of two different surface patterns formation by deposition of "neutral" (i.e. non-functionalized) and functionalized PLA-*b*-PEO copolymer nano-colloids on PLA surface.

Generally, the amphiphilic block copolymers form self-assemblies in selective solvents under dynamic equilibrium, i.e. with free exchange of unimers. Nevertheless, we have shown in our earlier studies that when appropriate molecular parameters of PLA-*b*-PEO block copolymer and the solvent are selected, the formed self-assembly can be brought into a state of a relatively kinetically stable nano-colloid. In such case, the unimer exchange is minimized, however, with the copolymer

core segments still not being completely frozen in a glassy state (Popelka *et al.* 2005). Thus, taking advantage of anchoring interactions between the PLA surface and the PLA chains in the self-assembly core, the copolymer layer on the polylactide surface can be formed by the deposition of whole PLA-*b*-PEO self-assemblies. When the mixtures of colloids with a different functionality are used, a different surface pattern of functional groups can be obtained, either with functional groups randomly distributed or forming distinct clusters with a higher group density. The corresponding modification scheme is shown in Figure 1.

To verify the different patterns, we applied the method based on visualization of streptavidin-labeled nanospheres bound to biotin at the end of RGDS peptide by scanning probe microscopy (Tresohlava *et al.* 2010, Knotek *et al.* 2013). The effect of surface patterns of RGDS peptide on cell attachment was evaluated in MG63 osteosarcoma cell culture.

Materials and Methods

Materials

KH_2PO_4 (Lach-Ner), $Na_2HPO_4.12H_2O$ (Lach-Ner), dimethyldichlorosilane (Fluka), acetanhydride (Lach-Ner), protected Fmoc amino acids (Fmoc-AA) and Fmoc-Lys(Biotin)-OH (Nova Biochem), chlorotrityl resin (Nova Biochem), 1-hydroxybenzotriazole (HOBT, Sigma-Aldrich), *N,N′*-diisopropylcarbodiimide (DIIC, Sigma-Aldrich), trifluoroacetic acid (TFA, Sigma-Aldrich), 1,2-ethanedithiol (EDT, Sigma-Aldrich), piperidin (Sigma-Aldrich), thioanisole (Sigma-Aldrich), pyridine (Lach-Ner), sodium 125-iodide solution for radiolabeling (LACOMED, CzechRepublic), TentaGel R Rink Amide resin (Rapp Polymere GmbH), α-aminoethyl-ω-hydroxy-poly(ethylene oxide) (M_w = 10000; JCS Biopolytech), streptavidin-labelled gold nanospheres (diameter 40 nm, $9x10^{10}$ particles per ml, Nanocs Inc., USA) and phosphate buffered saline tablets (PBS, Sigma) were used as purchased. Milli Q deionized water was used for all aqueous solutions and procedures. *N,N*-dimethyl-formamide (DMF, Lach-Ner) was distilled under vacuum in presence of ninhydrin. Tin(II) 2-ethylhexanoate ($Sn(Oct)_2$) (Aldrich) was distilled under vacuum and stored under inert gas at –18 °C. DL-lactide (Aldrich) was used freshly re-crystallized from a mixture of ethyl acetate and toluene. *N*-succinimidyl-3-maleimidopropionate was synthesized as described in Nielson and Buchardt (1991) and purified by repeated crystallization from isopropylalcohol.

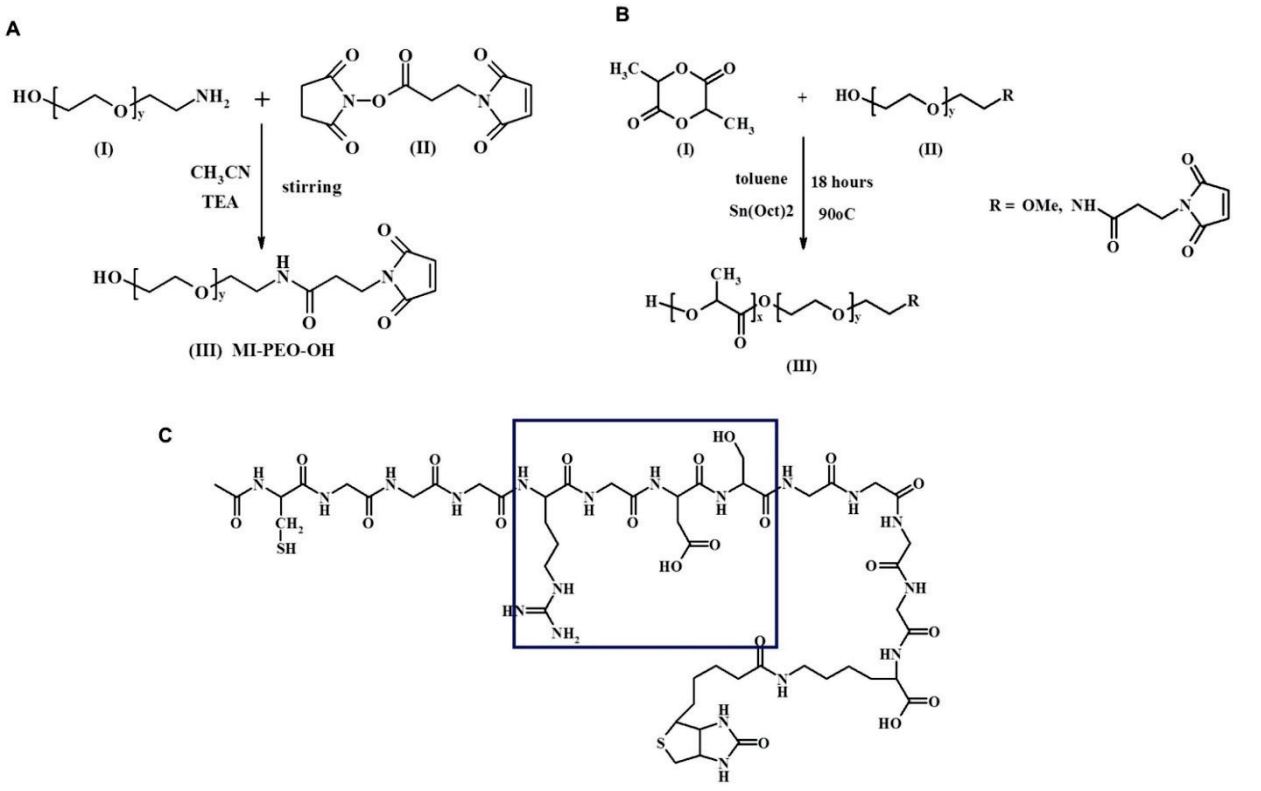

Fig. 2. Synthesis of **(A)** poly(ethylene oxide) with maleimide functional group (MI-PEO-OH) and **(B)** functional poly(lactide)-*block*-poly(ethylene oxide) copolymers. The structure of peptide containing RGDS sequence (framed), flanked on both sides with oligoG spacer, and thiol (Cys) and biotin end groups **(C)**.

Polymer synthesis

Poly(L-lactide) (PLLA) was synthesized by ring opening polymerization of L-lactide as described in Tresohlava *et al.* (2010).

α-Maleimide-ω-hydroxy-poly(ethylene oxide) (MI-PEO-OH): α-Amino-ω-hydroxy-poly(ethylene oxide) (I, Fig. 2A) (778 mg, $7.8x10^{-5}$ mol NH_2) and N-succinimidyl-3-maleimidopropionate (II) (29 mg, $11x10^{-5}$ mol) were dissolved in acetonitrile (12 ml) and triethylamine (16 µl, $7.8x10^{-5}$ mol) was added. The reaction was maintained for 2 h at 90 °C under reflux and then carried out at room temperature for another 20 h while stirring. The reaction product was poured into diethyl ether and the precipitated polymer was isolated by filtration. The dried product was stored under inert gas at –18 °C.

PLA-PEO-MI (MI-E10/L8), PLA-PEO-OMe (E10/L8, "neutral" copolymer) (Fig. 2B): Di-block copolymers composed of polylactide and poly(ethylene oxide) with either maleimide- or methoxy- group at the end of PEO block and with matching PLA and PEO block lengths were prepared by controlled ring opening polymerization of lactide (I) in toluene using heterobifunctional poly(ethylene oxide)s (II), i.e. MI-PEO-OH or MeOH-PEO-OH, as macroinitiators and $Sn(Oct)_2$ as a catalyst. The polymerization was carried out under the condition as described in Tresohlava *et al.* (2010). The dried products (III) were stored under inert gas at –18 °C.

Peptide synthesis

The model peptide Ac-CGGGGGGW-OH (data not shown) and Ac-CGGGRGDSGGGGK(biotin)-OH (Fig. 2C) peptide were synthesized by a stepwise solid-phase synthesis on chlorotrityl resin using standard Fmoc protocol according to Wellings and Atherton (1997) adapted according to Mackova *et al.* (2011). The coupling efficiency was monitored by visual test with Bromphenol Blue (Krchnak *et al.* 1988). The identity of the deprotected peptide was confirmed by MALDI-TOF analysis and reverse phase HPLC (data not shown).

Radiolabeled model peptide Ac-CGGGRGDSGGGY(^{125}I)-NH_2 was synthesized on TentaGel R Rink Amide resin using Fmoc SPPS strategy (Fields and Noble 1990) and procedure described previously (Kubinova *et al.* 2010). The identity of the peptide was confirmed by HPLC and MALDI-ToF MS analysis. The peptide was radiolabeled using chloramine T/ascorbic acid radiolabeling method (Hunter and Greenwood 1962) adapted for the conditions of the solid-phase peptide iodine radiolabeling (Mackova *et al.* 2011).

Biomimetic surface preparation

To mimic the surface of a bulk polylactide biomaterial, a PLLA film cast on a glass slide was used. Two type glass slides (circular, diameter 12 mm for AFM studies; rectangular 24x50 mm for cell culture experiments) was hydrophobized with dimethyl-dichlorosilane to facilitate a firm adhesion of the PLLA film. The model PLLA layers on the silanized glass slides were prepared according to protocol in Tresohlava *et al.* (2010). The functional PLA-*b*-PEO copolymer films were then cast on top of the PLLA film using spin-casting from solution mixtures containing different ratios of neutral (non-functionalized) and functionalized copolymers as follows.

Table 1. The composition of spin-cast copolymer solutions from dioxane/methanol mixed solvent (1/9 v/v, concentration 5 mg/ml) and corresponding surface codes of samples.

Surface code	Spin-cast solution composition
N^*	non-functionalized ("neutral") copolymer, E10/L8
$MI_R{}^*$	98 wt.% of E10/L8 + 2 wt.% of MI-E10/L8
$MI_C{}^*$	Mixture of two solutions: part A: part B in 9: 1 v/v ratio Part A: E10/L8 Part B: 80 wt.% of E10/L8 + 20 wt.% of MI-E10/L8

* The corresponding functional surface was denoted using subscripts R (=random) and C (=clusters) in surface (and solutions) codes, where MI refers to the surfaces with maleimide functionality. N refers to non-functionalized ("neutral") surface.

The PLA-*b*-PEO copolymer films on the PLLA sublayer were prepared by spin-casting of copolymer solutions using PWM32-PS-R790 spinner (Headway Research, Inc.), containing non-functionalized and maleimide-functionalized copolymer in selected compositions in dioxane/methanol mixed solvent

(1/9 v/v), as listed in Table 1. The slides with spin-cast copolymer films were annealed in a milliQ-water for 1 h at 60 °C and quenched by immersing into ice-cold milliQ-water afterwards. The coated slides were dried overnight at room temperature under vacuum (3 Pa) and stored under vacuum.

Grafting with RGDS peptide The glass slides containing a copolymer with maleimide group on the surface, i.e. MI_R and MI_C, were grafted with RGDS-peptide containing thiol end-group (Fig. 2C) *via* Michael addition. To this end, each sample was covered with 0.1 mg/ml peptide solution in phosphate buffer (pH~6.6, 70 µl on glass slide with diam. 12 mm, 750 µl on 24 x 50 mm rectangle coverslip) and the reaction was carried out at room temperature for 30 min. The samples were rinsed repeatedly with phosphate buffer and milliQ-water, dried for 16 h at room temperature under vacuum and stored under vacuum. The concentration of RGDS-peptide grafted on the maleimide surface was determined on glass slides with diam. 12 mm under similar condition using analogous radiolabeled peptide followed by radioactivity measurement.

Peptide labeling: surface reaction with streptavidin-labelled microspheres Circular glass slides with diameter of 12 mm grafted with biotin-tagged RGDS-peptide were exposed to the diluted dispersion of streptavidin-labeled Au nanospheres (Nanocs, Inc., 40 nm, dilution: 0.1 ml of original dispersion in 0.5 ml PBS). The reaction of microspheres with surfaces was performed by spreading a droplet of particle dispersion (0.1 ml) on the sample surface to react for 30 min at room temperature. The treated surfaces were rinsed thoroughly with PBS and MilliQ water, dried overnight at room temperature under vacuum and stored in a desiccator in darkness until characterization.

Characterization methods

Nuclear Magnetic Resonance Spectroscopy (NMR) ^{1}H NMR spectra of copolymers were acquired in $CDCl_3$ using high-resolution NMR spectrometer Bruker DPX300 at 330 K.

Size exclusion chromatography (SEC) was performed on Waters HPLC-SEC modular system according to description in Tresohlava *et al.* (2010). The number-average and weight-average molecular weight, M_n and M_w were determined.

Dynamic Light Scattering (DLS) All colloidal solutions of copolymers used for spin-casting were characterized by Dynamic Light Scattering on Zetasizer Nano instrument (ZEN 3600, Malvern, UK) at scattering angle θ=173° at 25 °C. The data were evaluated in DTS (Nano) program and the particle sizes (R_H) and size distribution (PdI) were determined by cumulant analysis.

Contact Angle Measurements (CAM) The material/water/air contact angle was determined by dynamic measurement based on sessile drop method and drop shape profile analysis on the Contact Angle Measuring System OCA_20 (Dataphysics, Germany) using SCA 20 software. At least 12 independent measurements were used for the evaluation. The advancing contact angle, θ_A, and receding contact angle, θ_R, were determined.

Atomic force microscopy (AFM), Atomic force acoustic microscopy (AFAM) AFM, AFAM and Phase Imaging measurements were carried out using Solver Pro M Atomic Force Microscope (NT-MDT; Russia). High-resolution silicon cantilevers CSG-01 (NT-MDT, typical k=0.03 Nm^{-1}) and acoustic transducer AFAM03 for the sample enforcing out-of-plane vibrations were used for measurements in AFAM mode. All AFAM measurements were realized on the descending slope of the first resonance mode. The probe HA_NC (NT-MDT, typical f=185 kHz, k=4.6 Nm^{-1}) was used for the Phase Imaging. The images were recorded at scan frequency 0.5-1 Hz with a resolution 256 × 256 pixels.

Determination of surface peptide concentration – radioactivity measurement Sample activities were directly measured using a Bqmetr 4 ionization chamber (Empos Ltd., Czech Republic) and a NaI/Tl SpectroAnalyzer, (AccuSync Medical Research Corporation, Milford CT 06460, USA).

Cell culture MG63, the human osteosarcoma cell line, was used to assess the patterned substrates. The glass coverslips (rectangular, 2.4 x 5.0 cm) coated with a PLLA base film and the copolymer surfaces MI_R-RGDS and MI_C-RGDS were sterilized by exposure to UV-light for 90 s. Each coverslip was then seeded with $2x10^5$ cells in 0.5 ml of DMEM media supplemented with 10 % fetal bovine serum (FBS) (Fisher, UK), 1 % L-glutamine and 1 % antibiotic-antimycotic solution (Sigma, UK). As a control sample, the neutral copolymer surface N, void of peptide ligands, was used. The static

culture group was kept in incubator at 37 °C and 5 % CO_2 for whole culture period. The cell morphology was taken in regular time intervals by a light microscopy (Olympus, UK). The dynamic culture group was cultured at static condition for 72 h first. After that, the samples were transferred to the four-point bending system, which applied cyclic unidirectional tensile strain to the cells at physiological level as described by Peake *at al.* (2000). The mechanical stimulation was applied to the coverslips for 1 h with 1 Hz frequency. After the mechanical loading, the samples were left undisturbed for 24 h post-culture prior to cell fixing and staining (Yang *et al.* 2004). The specimens from dynamic experiment were fixed by 4 % formalin and stained by a phalloidin-TRITC dye for actin filament following the manufacturer's protocol. The actin filaments were examined by staining of fibrillar actin and viewed through a confocal microscope (Olympus, FV 1000, UK).

Table 2. Molecular parameters of di-block copolymers.

Copolymer	Mn (PEO)[1]	Mn (PDLLA)[2]	Mn (total)	Mn/Mw[1]	Functionality*
E10/L8	10000	7850	17850	1.15	-
MI-E10/L8	10000	8200	18200	1.17	90 %

[1]Determined by SEC; [2] Determined by ^{1}H NMR; * Percentage of PEO block end groups occupied with a functional group (i.e. maleimidyl-) as determined by ^{1}H NMR.

Results

Polymer synthesis and characterization

High-molecular-weight homopolymer PLLA, and A-B di-block copolymers composed of polylactide (PLA) and poly(ethylene oxide) (PEO) blocks with matching block lengths and with either methoxy or maleimide functional groups at the end of the PEO block, were synthesized by ring-opening polymerization. The copolymers were characterized by ^{1}H NMR spectroscopy and size exclusion chromatography (SEC) (Table 2). The PLA-*b*-PEO copolymer with methoxy group at the end of PEO block, E10/L8, with matching PEO and PLA block lengths, was used as a non-adhesive, "neutral" background of functional copolymer when creating biomimetic surfaces. The molecular parameters of copolymers, i.e. the ratio of PEO and PLA blocks, have been calculated from ^{1}H NMR spectra using the ratio of integrated peaks of methylene protons of PEO unit (3.54 ppm) and that of PLA-methine proton (5.2 ppm).

The copolymer with maleimide-group at the end of PEO block, MI-E10/L8 was prepared *via* the reaction of *N*-succinimidyl-3-maleimidopropionate with amino group of α-amino-ω-hydroxy-PEO chain prior to the polymerization of lactide (Fig. 2A). The maleimide group was identified at 6.7 ppm in ^{1}H NMR spectrum as signal of two ethylene protons from double bond of maleimide cycle and at 2.5 ppm in the spectrum as signal of two methylene protons from the maleimido-propionate chain in α position to the amide bond.

Table 3. Water/air contact angles with Standard Deviation of the original and the modified PLLA surfaces.

			Grafted with RGDS peptide	
Surface	θ_A	θ_R	θ_A	θ_R
Silanized glass slides	98±1	68±2	-	-
PLLA film	82±1	68±1	-	-
N	56±2	15±3	-	-
MI_R	55±2	14±2	63±7	20±5
MI_C	54±1	13±2	39±3	9±2

N refers to neutral, i.e. non-functionalized surface, MI_R to surface with randomly distributed maleimide functional groups and MI_C to surface with maleimide group in clusters.

The hydrodynamic parameter and stability of colloidal particles formed by the copolymers in dioxane/methanol mixture solvent (1/9 v/v) were characterized by dynamic light scattering (DLS) on Malvern Zetasizer. The hydrodynamic radius (R_H) and the polydispersity index were determined for each type of the copolymer solution using cumulant analysis defined in International Standard ISO13321 (1996). In all cases, slightly opalescent colloidal solutions contained approx. 120 nm particles with a narrow particle size distribution, i.e. R_H = 60.0±2.0 and PdI = 0.2, regardless the composition of the solution.

Surface properties – wettability

The changes in surface wettability upon the deposition of the block copolymer colloids were used as a fast tool for monitoring the effect of the surface modification. The average contact angle values determined by dynamic contact angle measurement are presented in Table 3.

The average value of the contact angle for the PLLA support corresponds well with the values for PLLA obtained in our previous study (Tresohlava *et al.* 2010) and is in good agreement with values published elsewhere (Kobori *et al.* 2004). As it was expected, the presence of top-coating from PLA-*b*-PEO copolymers resulted in a significant decrease in the contact angles with respect to values for the underlying PLLA film, indicating the hydrophilic character of the copolymer coated surfaces. The addition of a fraction of maleimide-functionalized copolymer to the "neutral" PLA-*b*-PEO copolymer did not change the surface wettability significantly. It indicates that the presence of a small fraction (2 %) of maleimide functional groups has no significant effect on the gross physical surface properties. Finally, the hydrophilicity of the copolymer surfaces carrying maleimide group did not change significantly after grafting surfaces with RGDS peptide (Table 3).

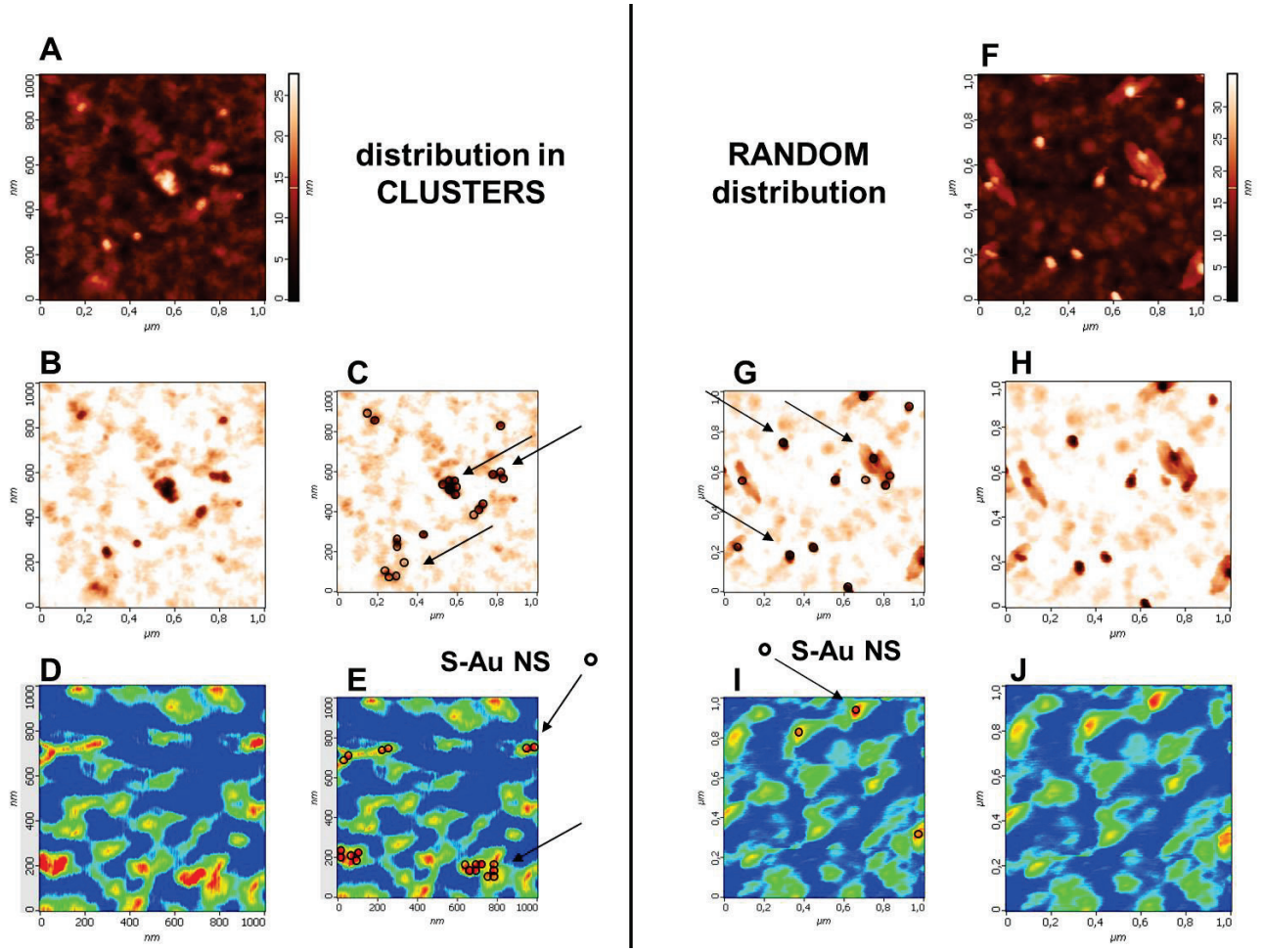

Fig. 3. Topographical **(A, F)** and phase **(B, C, G, H)** tapping mode AFM images and AFAM images **(D, E, I, J)**, all 1 x 1 μm, of S-Au NS (gold nanospheres) on PLA-*b*-PEO copolymer surface grafted with RGDS-peptide in organized and random distribution. The nanospheres bound to RGDS peptide on the surface are highlighted by black circle as depicted by black arrow in C, E, G, I.

Functional group detection and surface topography

The surface topography and the distribution of functional groups were examined by combining several modes of scanning probe microscopy (SPM), such as atomic force microscopy (AFM) in semi-contact mode and atomic force acoustic microscopy (AFAM). To visualize the localization of the RGDS-peptide grafted to the end of PEO block, the surfaces were tagged with streptavidin-modified gold nanospheres (S-Au NS), taking advantage of specific binding of streptavidin to the biotin group attached to the end RGDS-peptide. The tagging procedure with dispersion of S-Au NS was also applied to the "neutral" copolymer surface N, which was used as a reference sample.

Figure 3 shows the representative AFM images of copolymer surfaces tagged with S-Au NS. Using soft tips for phase imaging the nanospheres were identified as dark beads. As expected, the neutral copolymer surface N (data not shown), void of biotin groups, remained unchanged with essentially no spheres bound. It provides evidence that there is no significant non-specific adsorption of the nanospheric marker on the copolymer surface itself.

Figures 3A and 3F show copolymer surfaces grafted with peptide which were tagged with S-Au NS in a topography mode. Together with corresponding phase images of the same area in Figures 3B and 3H, they compare the surface topography pattern of MI_C-RGDS and MI_R-RGDS surfaces. These surfaces were prepared by different coating procedures leading either to the clustered or random distribution of functional groups and the grafted peptide. Although the fine structure of the copolymer surfaces was not significantly different, on the MI_R-RGDS copolymer surface, the most frequently only single nanospheres, randomly distributed over the surface were detected, with a typical picture in phase image mode shown in Figure 3H. The random distribution of biotin-labeled peptides, visualized as separate, mostly single S-Au NS was also confirmed by AFAM measurements on another MI_R-RGDS sample (Fig. 3J), in which the nanospheres are represented by red spots. Likewise as in Figure 3C, the detected S-Au NS can be highlighted by black circles of the corresponding size both in phase image (Fig. 3G) and in acoustic mode (Fig. 3I).

Contrary to that, on the MI_C-RGDS copolymer surface, formed from a mixture of separately made void/neutral and functional micelles, the most of S-Au NS were detected in distinct agglomerates, typically containing several nanospheres. The clustered pattern of S-Au NS, was confirmed by both, phase imaging AFM (Fig. 3B) and AFAM (Fig. 3D) mode (in a highlighted view Figure 3C and Figure 3E), respectively.

The effect of surface pattern on the initial cell adhesion

The preliminary cell experiments were performed to study the effect of different surface distribution of RGDS-peptide on copolymer surface otherwise with similar overall peptide concentration on the cell adhesion and spreading. The studied surfaces, prepared as thin films on glass coverslips, were transparent, which allowed the cells to be viewed by light microscopy.

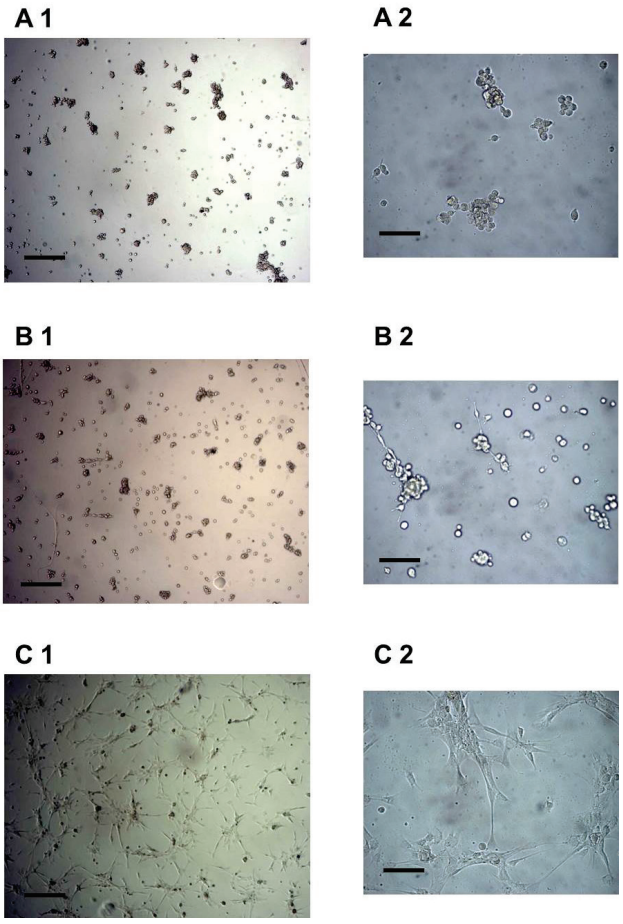

Fig. 4. Light micrographs of MG63 cells cultured statically on neutral copolymer surface N **(A1, A2)**; on copolymer surface with random distribution of RGDS-peptide on MI_R **(B1, B2)**; and on copolymer surface with RGDS-peptide distributed in clusters on MI_C **(C1, C2)**. The pictures were taken after 18 hours since seeding with cells. Scale bar in panel 1 is 80 μm, and panel 2 is 20 μm.

The results from MG63 cell culture are shown in Figure 4 for static cultivation and in Figure 5 for the loading system. On the unmodified PLLA film cells

adhered partially, some cells were stretched, but the major fraction of cells did not adhere properly and retained a spheroidal shape (not shown). On the surface N, i.e. void of RGDS peptide, the cells exhibited different morphology (Fig. 4A) indicating poor attachment and formation of multi-layered colonies retaining the spherical cell shape. The initial attachment of MG63 cells to MI_R-RGDS and MI_C-RGDS, i.e. surfaces with different surface pattern of RGDS peptide as it proved by AFM (Fig. 3) but with similar peptide concentration 8.5 x 10^{-12} mol/cm^2, is depicted in Figures 4B and 4C. The surface with a random distribution of RGDS-peptide, MI_R-RGDS, as depicted in Figure 4B, exhibited poor cell adhesion, with only few cells adhering properly. On the other hand, the cells on the surface with RGDS-peptide in clusters (Fig. 4C), MI_C-RGDS, were typically well spread, indicating good attachment. The micrographs in Figure 4 A1, B1 and C1 taken in low magnification show that the described cell response is general to entire sample surface and cannot be attributed only to some local surface defects/characteristics.

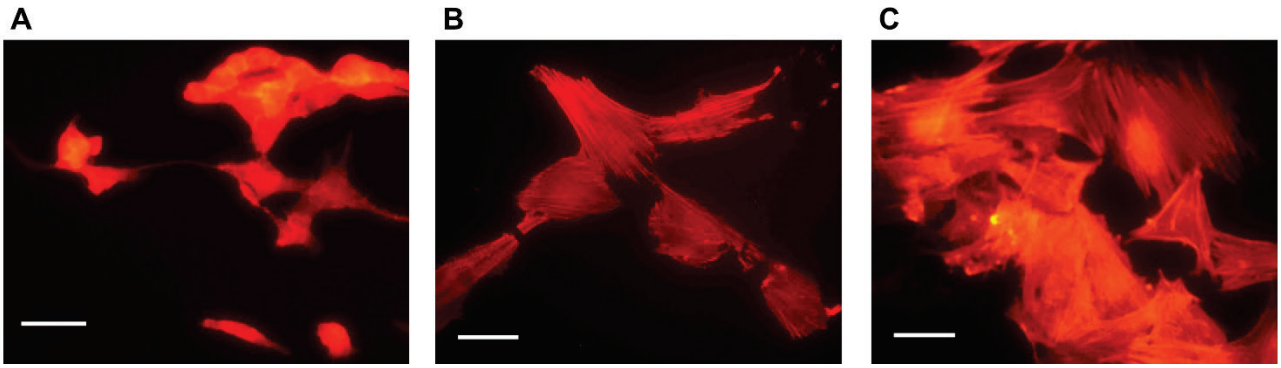

Fig. 5. The fluorescence micrographs of MG63 cells after mechanical loading on neutral copolymer surface N **(A)**; on copolymer surface with random distribution of RGDS-peptide on MI_R **(B)**; and on copolymer surface with RGDS-peptide distributed in clusters on MI_C **(C)**. Scale bar is 15 μm.

The effect of the surface pattern on MG63 cell line after mechanical stimulation evaluated as actin expression is depicted in Figure 5. The cells on the surface N remained round, aggregated in clusters with no actin filaments (Figure 5A) indicating poor cell adhesion. On the other hand, more evident expression of actin filaments was visible in both RGDS-peptide containing surfaces in Figures 5B and 5C. MI_C-RGDS surface not only contained higher number of adhered cells but also the actin filaments exhibit proper cytoskeleton organization with clearly parallel orientation (Fig. 5C).

Discussion

PLA-*b*-PEO block copolymers were synthesized by conventional ring-opening polymerization of lactide monomer using $Sn(Oct)_2$ as a catalyst and heterobifunctional poly(ethylene oxide)s as macroinitiators (Figure 2B), enabling the control of molecular weight of growing polylactide block by the amount of lactide monomer in the reaction mixture. Two amphiphilic PLA-*b*-PEO block copolymers, i.e. E10/L8 and MI-E10/L8 with narrow molecular weight (Table 2) were synthesized and used for preparation of model surfaces.

Amphiphilic block copolymers in selective solvents can form molecular assemblies; particularly, di-block PLA-*b*-PEO copolymers can associate into core-shell nanoparticles (Popelka *et al.* 2005). In the mixed solvent in this study, where methanol was used as the prevailing solvent acting as a coagulant for PLA (Kharas *et al.* 1994) and as a good solvent for PEO, the core is formed from collapsed polylactide chains while the flexible poly(ethylene oxide) chains generate the particle shell. In our study, the molecular parameters of the copolymers, i.e. the length and ratio of PLA and PEO blocks, and the composition of selective solvents were selected to accomplish two main criteria. Firstly, the length of the PEO block should be sufficient to form a copolymer layer with a dense PEO brush conformation, in order to suppress a nonspecific protein adsorption. Based on our previous data (Popelka *et al.* 2007) the molecular weight of PEO blocks around 10000 was selected. Secondly, we aimed at relatively stable core-shell particles exhibiting a very low rate of unimer exchange and, at the same time, with their core not

completely frozen with PLA in glassy state. In this way, the core-shell particles are still able to unfold in contact with the PLA surface and adsorb as whole particles. The length of the PLA blocks was based on previous study dealing with dynamics of unimer exchange in PLA-*b*-PEO micellar systems (Popelka *et al.* 2005). Dynamics of the chain exchange between self-assembled micelles of PEO-PLA diblock copolymers studied by direct nonradiative excitation energy transfer led to estimated MW of PLA block in a range of 4000-8000. In the present study, using the block copolymers with the latter MW of PLLA, i.e. 8 kDa, and MW of PEO about 10 kDa, the relatively stable non-functionalized and maleimide-functionalized nano-particles about 120 nm were obtained and used for preparation of model surfaces. The deposition of such PLA-*b*-PEO self-assemblies resulted in hydrophilic surfaces compared to the PLLA surface (Table 3). It indicates that PEO is present in the top layer in the brush-like structure and is exposed to the aqueous environment.

The surfaces carrying maleimide group, i.e. MI_R and MI_C, were grafted with the RGDS peptide flanked with Cystein, i.e. thiol- group, and with a biotin moiety at the second side of the peptide chain. The peptide was grafted *via* Michael addition of thiol to the double bond of maleimide at the end of the PEO chain on the surface. The used method is very fast and selective when carried out under slightly acidic conditions (Roberts *et al.* 2002). The surfaces grafted with RGDS were then evaluated in MG63 cell culture and related to the surface pattern of functional group.

The spatial distribution of the grafted adhesive motif on the PLA surface has been visualized by our established AFM protocol with gold particles through biotin and streptavidin interaction (Knotek *et al.* 2013). AFM imaging of the MI_R-RGDS and MI_C-RGDS copolymer surfaces tagged with S-Au NS clearly demonstrated that using S-Au NS (diameter of about 40 nm) as a high-density marker against the background of the copolymer surface, together with a potential of AFM/AFAM techniques provided a sensitive tool for detection of individual functional groups and monitoring their distribution pattern over the surface.

We have assumed that a surface with randomly distributed functional groups could be obtained when we used colloids with a uniform composition of the particles, e.g. hybrid micelles composed of neutral E10/L8 copolymer together with functional MI-E10/L8 copolymer. On the other hand, a surface with clusters of functional groups could be obtained when the surface was formed using a mixture of colloids composed of neutral micelles solely form E10/L8 and hybrid micelles from E10/E8 with MI-E10/L8. Indeed, AFM observation proved that under selected conditions, at least part of the nano-colloidal particles remained associated when they interact with the surface and are deposited as whole particles, thus giving rise to clusters of functional groups on the surface (Fig. 3). The approximate size of the clusters detected by AFM (100 to 150 nm) corresponds well with the size of nano-colloids determined by DLS. The estimated typical distance of biomimetic groups in the clusters was 40 nm and smaller. Taking into account that 40 nm is the diameter of S-Au nanospheres, the biotin-labeled peptides separated by a distance significantly shorter than 40 nm would not be revealed as two S-Au nanospheres.

Based on the obtained AFM results we can assume that the number of functional groups in the cluster thus can be controlled by the ratio of the neutral and MI functionalized copolymers used for the colloid formation. Further, our up-to-date experiments indicate that the number of clusters per surface area unit can be controlled by ratio of the functionalized and “neutral” nano-colloids during the deposition procedure.

The attachment of cells to their support matrix is important in determining cell shape and in maintaining proper cell function and tissue integrity. The appropriate cell adhesion causes positional signals which direct cellular traffic and differentiation so the quality of this first phase will influence the cell’s capacity to proliferate and differentiate itself on contact with the implant, for example (Anselme 2000, El Haj *et al.* 2005). Therefore, our aim of this study was to test whether the alteration of adhesion ligands distribution can affect cell adhesion in static culture and dynamic environment, especially when applying tensile force to bone cells seeded on the surface of modified films.

The unmodified PLLA has rather hydrophobic character with a contact angle θ_A approx. 82 degrees. It can adsorb proteins from the serum in the culture media. Thus, the adhesion of the MG63 cells to PLLA was mediated primarily by nonspecific adsorption of proteins from the culture medium. There was no pattern and thus no control over the cell adhesion. The low cell affinity to the neutral PEO-PLA copolymer surface N (Fig. 4A) was in agreement with general assent, including our previous studies (Bacakova *et al.* 2007). Therefore, this surface can be considered as non-adhesive background with

a non-fouling feature, on which the effect of attached biomimetic groups can be revealed.

The maximum lateral spacing of RGDS molecules for efficient cell attachment, spreading and formation of focal adhesion was determined in the range of 58 to 73 nm (Arnold *et al.* 2004) for different cell types. The preferred cell attachment and spreading onto MI_C-RGDS compared to MI_R-RGDS surface is in good agreement with these observations. The more efficient cell attachment to MI_C-RGDS surface (Fig. 4C) can be explained by the distance of RGDS peptide chains in one cluster estimated as approx. 40 nm (Figs 3C and 3E). On the other hand, the spacing between individual RGDS molecules on the MI_R-RGDS surface with randomly distributed RGDS peptide, is higher than 100 nm (Figs 3G and 3I). This distance was reported by Arnold *et al.* (2004) to be insufficient for proper cell adhesion.

The main focus of this study is to investigate whether substrates treated by RGDS in specific pattern can stabilize the cell attachment because the enhancement effect of RGDS on cells under static culture condition have been widely studied. However, their effect on dynamic culture is less studied. A mechanical stress could detach cells from the substrate if the cells did not adhere properly on the substrates, and the cellular metabolism would not be affected through mechano-transduction. Therefore, a four-point bending bioreactor system was applied to examine whether the RGDS peptides grafted to the surface was bound strongly enough to sustain cell attachment in dynamic culture conditions. Importantly, since the MG63 cells were present on the surfaces with both modifications, i.e. with random or cluster topography of RGDS motif (Figs 5B and 5C), after mechanical loading, we can conclude that the proposed pattern modification is sufficiently stable for applying tensile strain in order to mimic physiological conditions. The beneficial effect of the organization of RGDS in clusters on cell adhesion and spreading (Fig. 5C) is revealed by comparison to the surface with random distribution of RGDS peptides (Fig. 5B) or neutral surface (Fig. 5A). The MI_C-RGDS surface contained higher number of adhered cells than the MI_R-RGDS with a random RGDS distribution. Further, the cells were well spread and more stretched, and actin filaments exhibited dense network cytoskeleton organization with clearly parallel orientation of actin fibers. Thus we can expect that, besides enhanced cell attachment, the proposed clustered RGDS distribution will provide that cells will trigger signal pathways resulting to proper cell proliferation, migration and differentiation.

Conclusions

The polylactide surfaces can be effectively modified by grafting of RGDS peptide into distinct patterns; random or cluster distribution through manipulation of the ratio of functionalized and non-functionalized nano-colloids of amphiphilic PLA-*b*-PEO di-block copolymers. The modified surfaces exhibited markedly increase of wettability compared to pure PLLA surface. AFM techniques have been proved as an effective method to illustrate the average RGDS peptide spatial distribution on the grafted surfaces through the visualization of streptavidin-labeled gold nanospheres bound to biotin groups at the end of RGDS peptide on the surface.

Both PLA-*b*-PEO copolymer surfaces grafted with RGDS peptide enhanced cell attachment with strong actin expression in dynamic culture environment, whilst the surfaces with RGDS peptides arranged in clusters exhibited better cell adhesion, manifesting as higher actin expression. On the contrary, the surface modification with PLA-*b*-PEO copolymer void of RGDS peptide prevented cell attachment. Thus, this technique offer a tool to deliver multifariously functionalized surfaces with respect to used cell types on 2D model substrates as well as on 3D scaffolds.

Conflict of Interest

There is no conflict of interest.

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