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Relationship between the fibroblastic behaviour and surface properties of RGD-immobilized PCL membranes

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Abstract In this study, poly-ε-caprolactone (PCL) membranes were modified with the cell adhesive peptide RGD by chemical immobilization technique. The roughness and hydrophilicity were increased after RGD immobilization and an improved cell attachment was observed.

1 Introduction

Poly-ε-caprolactone (PCL) is a biodegradable, soft and hard tissue compatible material and it is considered as a potential substrate for wide applications, such as drug delivery systems, tissue-engineered skin, axonal regeneration and scaffolds for supporting fibroblast and osteoblast growth. Moreover, the interaction between cells and PCL can be further improved by immobilizing bioactive molecules on the surfaces. The Arg-Gly-Asp (RGD) sequence of fibronectin, is by far the most effective and most often employed peptide sequence for stimulated cell adhesion on synthetic materials [1]. In the presented study, the cell binding RGD domain is immobilized on PCL surfaces by using chemical immobilization method [2] for its possible use in tissue engineering applications.

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2 Materials and methods

2.1 Materials

Poly(ε-caprolactone) (PCL, average M_w ~65,000, average M_n ~45,000) pellets were obtained from Aldrich (Germany). Pure hexamethylene diisocyanate (HMDI) and 4-dimethyl amino pyridine (DMAP) were purchased from Fluka (Germany). Disuccinimidyl suberate (DSS), dimethyl formamide (DMF) and Arginine-Glycine-Aspartic acid, Arg-Gly-Asp (RGD, MW: 346.3 g $gmol^{-1}$) were obtained from Sigma (Germany). All the materials were used without further purification.

2.2 Preparation and biomodification of PCL membranes

Model surfaces of PCL (Aldrich, Germany) were prepared in membrane form by solvent casting method. The biomodification of PCL membranes were realized at four stages. At the first stage, isocyanation was performed using hexamethylene diisocyanate (HMDI, Aldrich, Germany) (10%, v/v) in dimethyl formamide (DMF, Aldrich, Germany) in the presence of dimethyl amino pyridine (DMAP, Aldrich, Germany) (0.5%, w/v). This stage is followed by a surface hydrolysis to obtain amino groups by immersing the membranes in 1N NaOH aqueous solution, which were in turn activated in the presence of disuccinimidyl suberate (DSS, Aldrich, Germany) at the third stage. At the last stage, RGD immobilization was realized.

2.3 Characterization of unmodified and RGD-modified PCL membranes

2.3.1 X-ray photoelectron spectroscopy (XPS)

XPS analysis was performed by using a PHI 5600 Multi Technique Spectrometer equipped with dual Al/Mg anode, hemispherical analyser and electrostatic lens system (Omni Focus III). Al K$\alpha_{1,2}$ radiation with pass energies of 187.5 eV for survey scans and 11.75 eV for the detailed scans was used. The photoelectron peaks were analysed by using a curve-fitting routine based onto Gaussian shapes and a linear Shirley background subtraction approximation. Binding energies (BEs) of all the spectra were referenced to the intrinsic hydrocarbon like C1s peak assumed at 285.0 eV.

2.3.2 Atomic force microscopy (AFM)

The surface micro-topography and the morphology of the surfaces were measured with a Multimode/Nanoscope IIIA Atomic Force Microscope (Veeco, USA) in tapping mode in air with a standard silicon tip. The relative room humidity was 30% and the room temperature was 25°C. Data were acquired on square frames having edges of 10 μm, 1 μm and 350 nm. Images were recorded using height and phase-shift channels with 512 × 512 measurement points (pixels). Image processing was performed by using Nanoscope III software.

2.3.3 Contact-angle measurements

Measurements of static and dynamic water contact angle were performed by a half automatic video-based OCA apparatus (Dataphysics, USA) at 25°C and 35% relative humidity. By using the sessile drop method, liquid drops of 2 μl of volume were applied on different zones of each sample surface and by digital image analysis the dynamic contact angles (θ_s) were measured on both sides of the two-dimensional projection of the droplet. Both Young and Wenzel water contact angle (WCA) were evaluated, in order to take in account the surface roughness effect. At least five measurements were made for each sample and then averaged.

2.4 Cell culture studies

Cell attachment was investigated by using L929 fibroblasts obtained from HUKUK Cell Line Collection (No: 92123004 Footh and Mouth Disease Institute, Turkey). Cell culture studies were carried out in parafilm lined 24-well tissue culture plates by using Dulbecco's modified Eagle's medium (DMEM, Sigma, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma) under CO_2 (5%) atmosphere at 37°C. Unmodified and RGD-modified PCL membranes were cut in circular pieces, submerged into parafilm-lined (parafilms soaked in 95% ethanol before for sterilization) 24-well tissue culture plates and sterilized by UV irradiation for 15 min. Cells were seeded at a density of 2×10^4 cells per well in 1 ml of cell suspension. For investigating the adhesion of L929 fibroblasts, the medium was aspirated at short culture times within 24 h and the non-adhered cells were counted in the medium with a Neubauer hemocytometer [3]. The results are given as the percentage of attached cells. The cell attachment rate of L929 fibroblasts and the time required for 50% of cells to attach on the membranes were also evaluated for unmodified and RGD modified PCL membranes. Tissue culture plates were used as the control.

3 Results and discussion

The results obtained from XPS analysis showed the effective RGD immobilization by the increase of nitrogen concentration at the surfaces of biomodified membranes. The results obtained from detailed scans giving the surface atomic composition are summarized in Table 1.

AFM studies showed that the surface morphology of the membranes was significantly changed after biomodification process. In particular, the surface roughness parameters R_{ms} and R_a given in Table 2 show that RGD-modified surfaces exhibit a strong roughening effect ∼2.5 times more rough than the unmodified-PCL membranes.

This increase in roughness can be attributed to the chemical treatment during the immobilization procedure, also involving the removal of low molecular weight products from the surfaces. The AFM height images for unmodified-PCL and RGD-modified PCL membranes are given in Fig. 1.

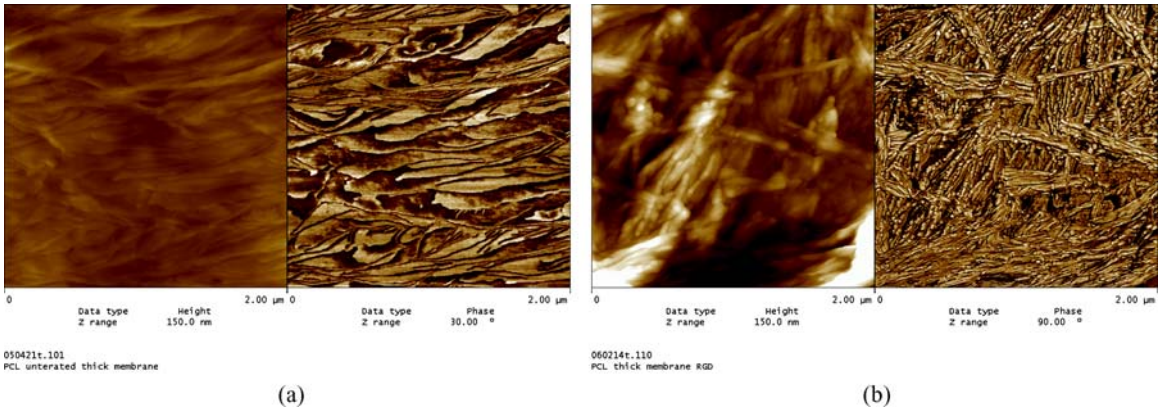
The results obtained from contact angle measurements reveal the increase of PCL surface wettability after biomodification with RGD. Young and Wenzel water contact angle (WCA) measurements are given in Table 3. The increase in

Table 1 XPS surface atomic concentrations of PCL membranes before and after biomodification

XPS surface atomic concentration	Membranes	
	Unmodified PCL	RGD-modified PCL
−O (at.%)	24.8	22.8
−N (at.%)	–	4.7
−C (at.%)	75.2	72.5

Table 2 R_{ms} and R_a values on 1 μm scale for unmodified and RGD-modified PCL surfaces

	R_{ms} (nm)	R_a (nm)
Unmodified PCL	10.39 ± 4.05	8.22 ± 3.64
RGD-modified PCL	24.77 ± 9.03	32.43 ± 9.58



(a) (b)

Fig. 1 AFM height and phase images of PCL membranes before (a) and after (b) RGD immobilization. Z scale = 150 nm

Table 3 The advancing WCA values of PCL membranes before and after biomodification

	Membranes	
Contact Angle	Unmodified PCL	RGD-modified PCL
Young WCA adv	63.9° ± 2.8°	55.1° ± 1.8°
Wenzel WCA adv	54.0° ± 3.0°	46.9° ± 2.0°

Table 4 The percentage of attached cells on unmodified and RGD-modified PCL membranes. Data are expressed as means of a representative of three similar experiments carried out in triplicate

	% Attached cells		
Time (h)	Unmodified- PCL	RGD-modified PCL	TCPS
2	12.5 ± 1.1	56.3 ± 0.6	87.5 ± 0.2
4	25.0 ± 2.0	75.0 ± 2.9	100.0
6	50.0 ± 0.9	81.5 ± 1.2	
8	81.3 ± 1.4	88.0 ± 0.5	
24	87.5 ± 0.7	90.0 ± 3.4	

hydrophilicity is in agreement with the measured roughness change.

The *in-vitro* cytocompatibility of unmodified and RGD-modified membranes was investigated by L929 mouse fibroblast cell culture. The results obtained from cell culture studies showed that the presence of RGD on PCL membrane triggered the initial cell attachment of fibroblasts on PCL membranes. The percentage of attached cells during 24 h culture is given in Table 4 for unmodified and RGD-modified membranes.

While the attachment rate on PCL membranes was calculated as $k = 0.140\ h^{-1}$, the attachment rate on RGD modified membranes was calculated as $k = 0.215\ h^{-1}$. More than 50% cells were attached on RGD-modified PCL membrane within 2 h. As a result, an improved initial cell attachment was observed on RGD immobilized PCL membranes.

4 Conclusion

In this study the PCL membranes were successfully modified by using chemical immobilization technique with cell adhesion motif RGD. The presence of RGD molecules on the surface affected the cell attachment rate and an improved cell adhesion was observed. The observed effect indicates that the grafting methods here employed allow RGD to maintain its biological functionality. Further applications in tissue engineering the same immobilization technique can be considered with PCL membranes and different cell adhesive motifs for specific interactions.

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