

SURFACE FUNCTIONALIZATION OF POLY(L-LACTIDE-CO-GLYCOLIDE) MEMBRANES WITH AMPHIPHILIC POLY(2-OXAZOLINE) FOR GUIDED TISSUE REGENERATION AND TREATMENT OF BONE TISSUE DEFECTS

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Abstract

The main challenge of this research was to functionalize the surface of poly(L-lactide-co-glycolide) (PLGA) membranes with amphiphilic poly(2-oxazoline) (POx) in order to change PLGA chemical state and properties. Poly(2-oxazolines) are very powerful polymers, which thanks to active pendant groups can be easily functionalized with biologically active molecules or peptides. The membranes were prepared by dissolving PLGA, POx, and poly(ethylene glycol) (PEG, 1000 Da) in methylene chloride (DCM), followed by PEG leaching. POx molecules were preferentially adsorbed at the interface PLGA-POx-PEG thanks to affinity to both hydrophilic (PEG) and hydrophobic (PLGA) chains. The properties of the membranes were characterized with Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and wettability tests. Cytocompatibility of the materials in contact with osteoblast-like MG-63 cells was studied by evaluation of cell viability (Alamar-Blue test), live/dead and phalloidin/DAPI staining. The results show that the presence of POx influenced topography of the PLGA membranes, but did not have an impact on their wettability. All membranes were found cytocompatible with model osteoblasts. Presence of POx resulted in better cell adhesion as shown by microscopic studies after fluorescence staining for nuclei and cytoskeleton actin filaments. In summary, one-step phase separation process between PLGA, PEG, and POx, dissolved in DCM followed by drying and PEG leaching resulted in cytocompatible PLGA membranes with immobilised POx, which might be considered for guided tissue regeneration technique in periodontology and in bone tissue engineering.

Keywords: poly(L-lactide-co-glycolide), poly(ethylene glycol), poly(2-oxazolines), phase separation, Fourier transform infrared spectroscopy (FTIR), osteoblast-like cells, guided tissue regeneration (GTR), bone tissue engineering

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Introduction

Impaired bone healing is a crucial clinical problem in orthopedics, oral and maxillofacial surgery, and periodontology. Medical devices enhancing bone repair and healing should be made of biomaterials with adequate properties, such as biocompatibility, bioactivity, osteoconduction, and biodegradation [1,2]. Particularly interesting form of biomaterials are membranes, which are widely used in artificial organs and diagnostic devices. They can be also used in periodontal applications and act as barriers in guided tissue regeneration (GTR) to protect bony defects from the invasion of connective tissue and mucous membrane, and assure adequate conditions for regeneration of bone tissue [1,3,4].

Poly(L-lactide-co-glycolide) (PLGA) has been often applied to create scaffolds for bone tissue engineering and membranes for periodontology [5]. Several strategies have been used to fabricate PLGA membranes, for example, porogen leaching, fibers bonding or phase separation [3,6]. A key feature of PLGA is its degradation by hydrolysis. The final degradation products of PLGA, i.e. lactic and glycolic acids are removed by natural metabolic pathways [5]. However, despite being biocompatible, clinical application of PLGA for bone regeneration and GTR is hampered by poor osteoconductivity and lack of cell adhesion motifs such as arginine-glycine-aspartic acid (RGD), which are beneficial for cell adhesion controlled by interaction with integrins [7].

Poly(2-oxazolines) (Pox) are amphiphilic, non-ionic polymers, which are synthesised *via* living cationic polymerization [8]. They are biocompatible and have been used in medical and drug delivery applications. They can be conjugated with drugs, peptides and proteins, e.g. RGD [9].

A successful approach in biomaterials science is to design the surface properties of approved materials for specific medical application. The main challenge in this area is the development of processing routes allowing for simple and efficient surface modification of complex shaped geometries. Thus, our research aimed at surface functionalization of PLGA membranes *via* phase separation between PLGA, water-soluble poly(ethylene glycol) (PEG) and amphiphilic bifunctional POx [10]. Physicochemical and biological properties of resulting membranes important from the point of view of periodontology and bone tissue engineering applications were analyzed and described in this paper.

Materials and Methods

Materials

The membranes were made from PLGA (85:15, 100 kDa, $d = 1.9$, synthesised at Centre of Polymer and Carbon Materials, Polish Academy of Sciences in Zabrze, Poland) [11]. As a solvent DCM and as a pore former PEG (1000 Da), both from Sigma-Aldrich, were used. As an amphiphilic POx molecule poly(2-methyl-2-oxazoline-b-2-butyl-2-oxazoline) and more specifically methyl-P[MeOx₃₇-b-BuOx₁₀-b-MeOx₂₀-piperidine(P2—P2) (Mn = 8 kDa, $d = 1.14$, synthesised at Technische Universität Dresden, Germany) [12] was used.

Manufacturing methods

PLGA and PEG were co-dissolved in DCM at a concentration of 10% wt/vol and 1 wt% POx in respect to PLGA was added. The membranes PLGA-POx were obtained by solvent casting on glass Petri dishes, followed by air drying for 24 h and vacuum drying for 24 h. Afterwards, PLGA-POx-PEG blends were washed in purified water for 5 days; water was exchanged every 30 min during day 1 and every 2 h during days from 2 to 5.

As references PLGA membranes and PLGA foils without POx were made by dissolving PLGA in DCM at a concentration of 10%, slip casting on Petri dishes, followed by air drying for 24 h and vacuum drying for 24 h.

Evaluation methods

FTIR in attenuated total reflection mode (ATR) with ZnSe crystal has been used for the characterization of main ingredients used in membranes preparation, i.e. PLGA, PEG and POx, as well as obtained PLGA-PEG-POx blend, PLGA-POx membrane and PLGA foil. FTIR spectra were recorded on Nicolet iS5-iD5 spectrometer with resolution of 4 cm^{-1} .

Water contact angle values were measured on DSA Mk2 (Krüss, Germany) using ultra-high purity water (UHQ-water, Pure-Lab, UK) droplets of $0.20\ \mu\text{l}$. Droplets were deposited on the surfaces at room temperature, and their images were recorded using a video camera and analyzed using an image analysis system (DSA software). The results were expressed as the average and standard deviation (S.D.) of 10 droplets deposited on the surface of the samples.

Microstructure of the samples was assessed with scanning electron microscopy (SEM, Nova Nano SEM 200, USA, secondary electron mode).

Cytocompatibility tests were performed on osteoblast-like MG-63 cells. Prior to the experiment, the samples were placed in the wells of 24-well plates, incubated in 70% ethanol for 10 min, then the samples were transferred to 48-well plates and left under the laminar hood and UV lamp overnight for sterilization. 8×10^3 cells suspended in 1 ml cell culture medium (EMEM, ATCC, USA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 0.1% amino acids and sodium pyruvate (PAA, Germany) were poured on the samples and cells were cultured at 37°C under a humidified atmosphere with 5.0% CO_2 for 24 and 72 h.

Cell viability was evaluated using AlamarBlue reagent (In Vitro Toxicology Assay Kit, resazurin based, Sigma-Aldrich) and live/dead staining using 1 ml of PBS supplemented with $1\ \mu\text{l}$ (1 mg/ml) of calcein AM (Sigma) and $1\ \mu\text{l}$ (1 mg/ml) of propidium iodide (Sigma). The cells were also stained for phalloidin/DAPI to visualize actin fibers and nuclei, respectively. The samples were washed twice in PBS and observed under a fluorescence microscope (Zeiss Axiovert 40, Carl Zeiss, Germany). Statistical analysis was performed using a one-way analysis of variance (one-way ANOVA). Significant differences were assumed at $*p < 0.05$. The results were presented as mean \pm S.D.

Results and Discussion

FIG. 1 shows FTIR-ATR spectra of main ingredients: PLGA, PEG 1000 and POx. In PLGA spectrum a strong band at 1746 cm^{-1} is present, which can be assigned to stretching vibration of carbonyl groups. There are also bands between 1300 and 1150 cm^{-1} originating from asymmetric and symmetric C-C(=O)-O stretching vibrations [3,13].

In the spectrum of PEG characteristic bands are visible: at 3400 cm^{-1} due to stretching vibrations of water molecules, at around 2880 cm^{-1} and in the range of $1450\text{--}1240\text{ cm}^{-1}$ due to stretching and deformational vibrations of hydrocarbon in CH_2 groups, respectively. Moreover, bands in the range of $1000\text{--}1200\text{ cm}^{-1}$ are attributed to stretching vibrations of CO and COC groups. At lower wavenumbers in the range of $840\text{--}940\text{ cm}^{-1}$ bands assigned to bending vibrations of hydrocarbon groups in CH_2 are observed [3,14].

In the spectrum of POx there are bands at 1130 cm^{-1} and 1621 cm^{-1} . The band in the range $1700\text{--}1600\text{ cm}^{-1}$ is characteristic for poly(2-oxazolines) and is due to stretching vibrations of C=O and C-N groups [15].

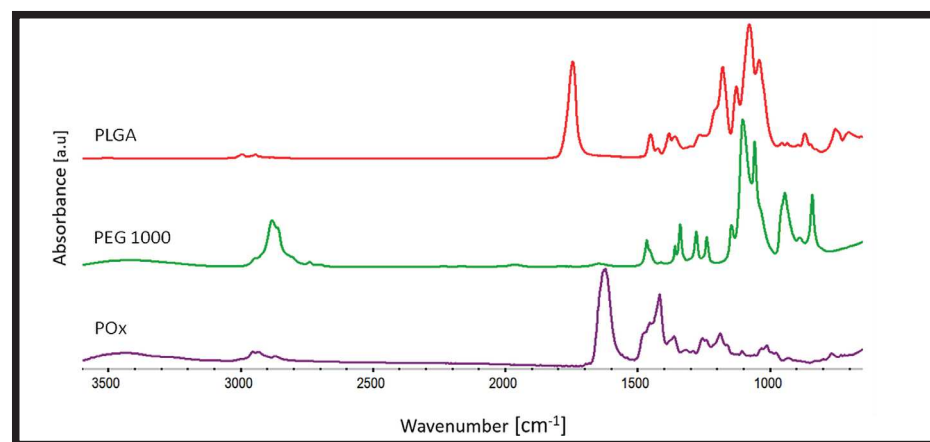


FIG. 1. FTIR-ATR spectra of main ingredients: PLGA, PEG 1000, and POx.

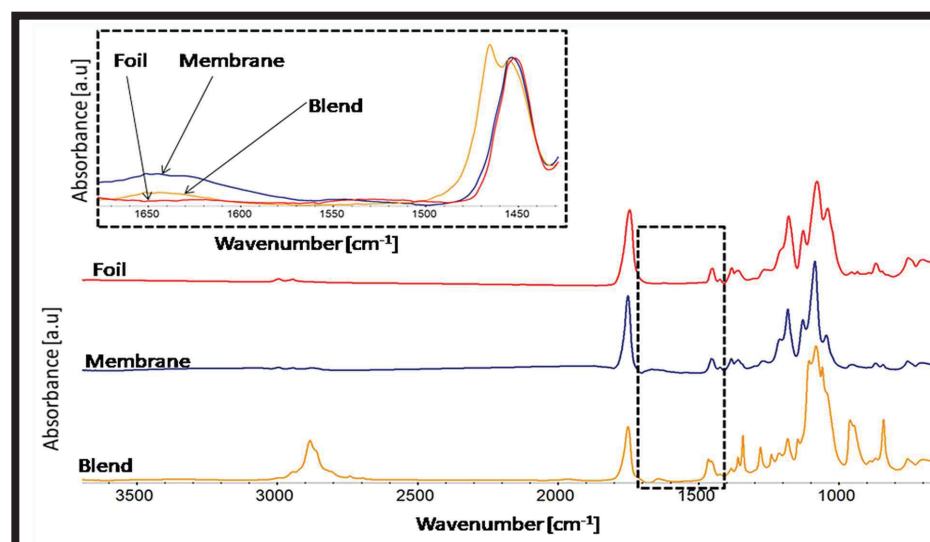


FIG. 2. FTIR-ATR spectra of PLGA foil (Foil), PLGA-POx membrane (Membrane), and PLGA-POx-PEG blend (Blend). In the insert respective spectra in the range of $1400\text{--}1690\text{ cm}^{-1}$ are shown for better visualization of differences.

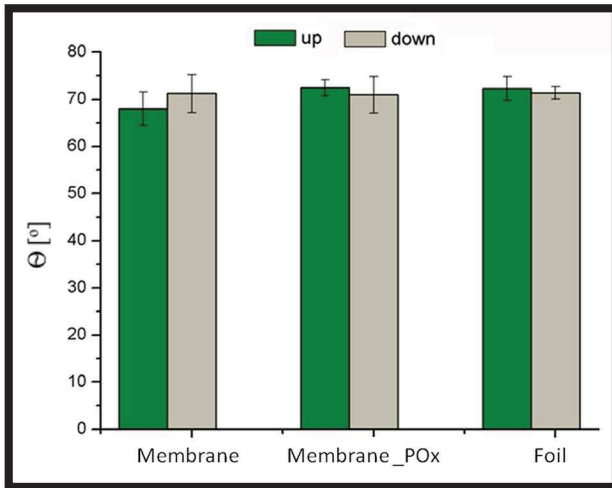


FIG. 3. Water contact angle values of PLGA membrane, PLGA membrane modified with POx, and PLGA foil: up / down – surfaces in contact with air / glass during preparation. No significant differences according to ANOVA.

As it is shown in FIG. 2 PLGA-POx-PEG blend contains all peaks characteristic for all ingredients: at 1746 cm^{-1} for PLGA, at around 2880 cm^{-1} for PEG, and at around 1620 cm^{-1} for poly(2-oxazolines). The membrane contains all bands characteristic for PLGA foil (strong band at 1746 cm^{-1} , due to stretching vibration of carbonyl groups and bands between 1300 and 1150 cm^{-1} from asymmetric and symmetric C-C(=O)-O stretching vibrations) [9]. The peaks of medium intensity at 2950 and 1370 cm^{-1} are attributed to C-H stretching and bending modes in PLGA structure, respectively. Moreover, a weak and wide band at around 1620 cm^{-1} from C=O and C-N stretching vibrations, which is characteristic for poly(2-oxazoline) structure is visible [15]. This band is not observed in the spectrum PLGA foil, what is an indirect proof that POx is immobilized on the surface of the membranes.

The results of wettability of PLGA membrane, PLGA membrane modified with POx and PLGA foil, both lower and upper surfaces, i.e. being in contact with glass and air during preparation are shown in FIG. 3. It is apparent that all analysed materials are hydrophilic with the water contact angle of about 70° . No significant differences were found between all studied materials.

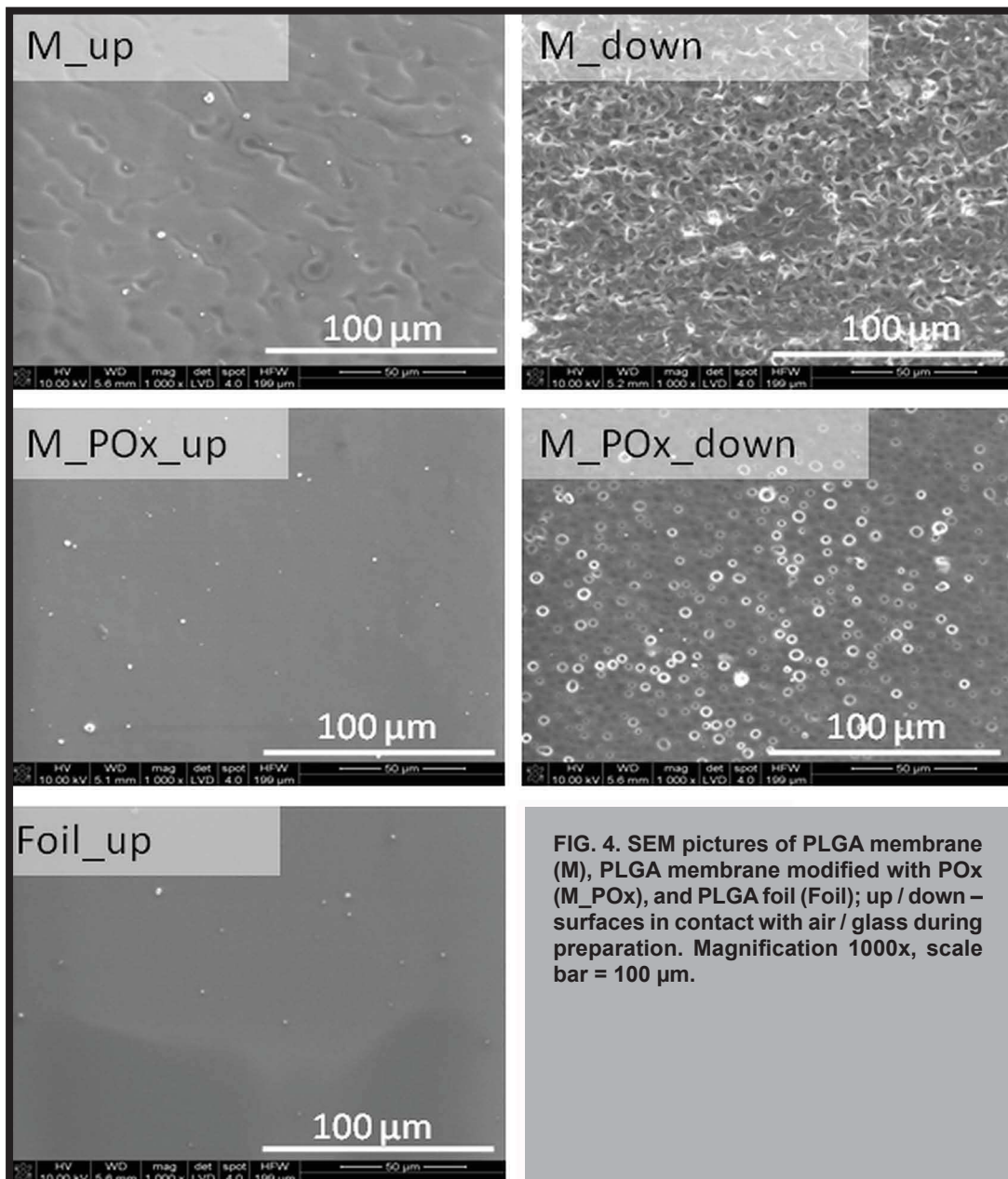


FIG. 4. SEM pictures of PLGA membrane (M), PLGA membrane modified with POx (M_POx), and PLGA foil (Foil); up / down – surfaces in contact with air / glass during preparation. Magnification 1000x, scale bar = $100\text{ }\mu\text{m}$.

SEM results (FIG. 4) show that the air-cured (M_up, M_POx_up) and glass-cured (M_down, M_POx_down) surfaces of the membranes have different microstructure. Air-cured surfaces are smoother and non-porous, while glass-cured are porous. Thus, asymmetric membranes were obtained. Interestingly, the presence of POx in the membrane resulted in reduction in roughness on the air-cured surface and reduction in pore size and pore number on the glass-cured surface. PLGA foil was the smoothest among all studied materials.

To verify if PLGA membranes produced were compatible with cells *in vitro*, viability and morphology of MG-63 osteoblast-like cells were studied. The cells were cultured on both surfaces of two membranes: with and without POx and reference PLGA foil for 24 and 72 h (FIG. 5). The viability of the cells cultured for 24 h on all the membranes was the same or higher as compared to cell viability on control foil. The viability of the cells cultured for 72 h on all the membranes was significantly higher as compared to control foil. This suggests that the PLGA membranes are cytocompatible with model osteoblasts and that presence of POx does not have a negative impact on cells.

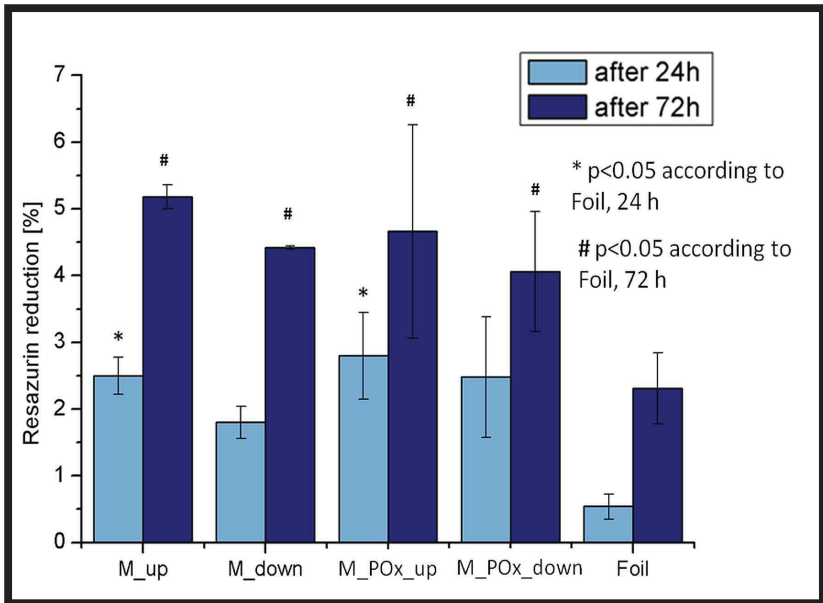


FIG. 5. MG-63 cell viability measured by resazurin reduction for PLGA membrane (M), PLGA membrane modified with POx (M_POx), and PLGA foil (Foil); up / down – surfaces in contact with air / glass during preparation. Statistical significance according to ANOVA at $p < 0.05$.

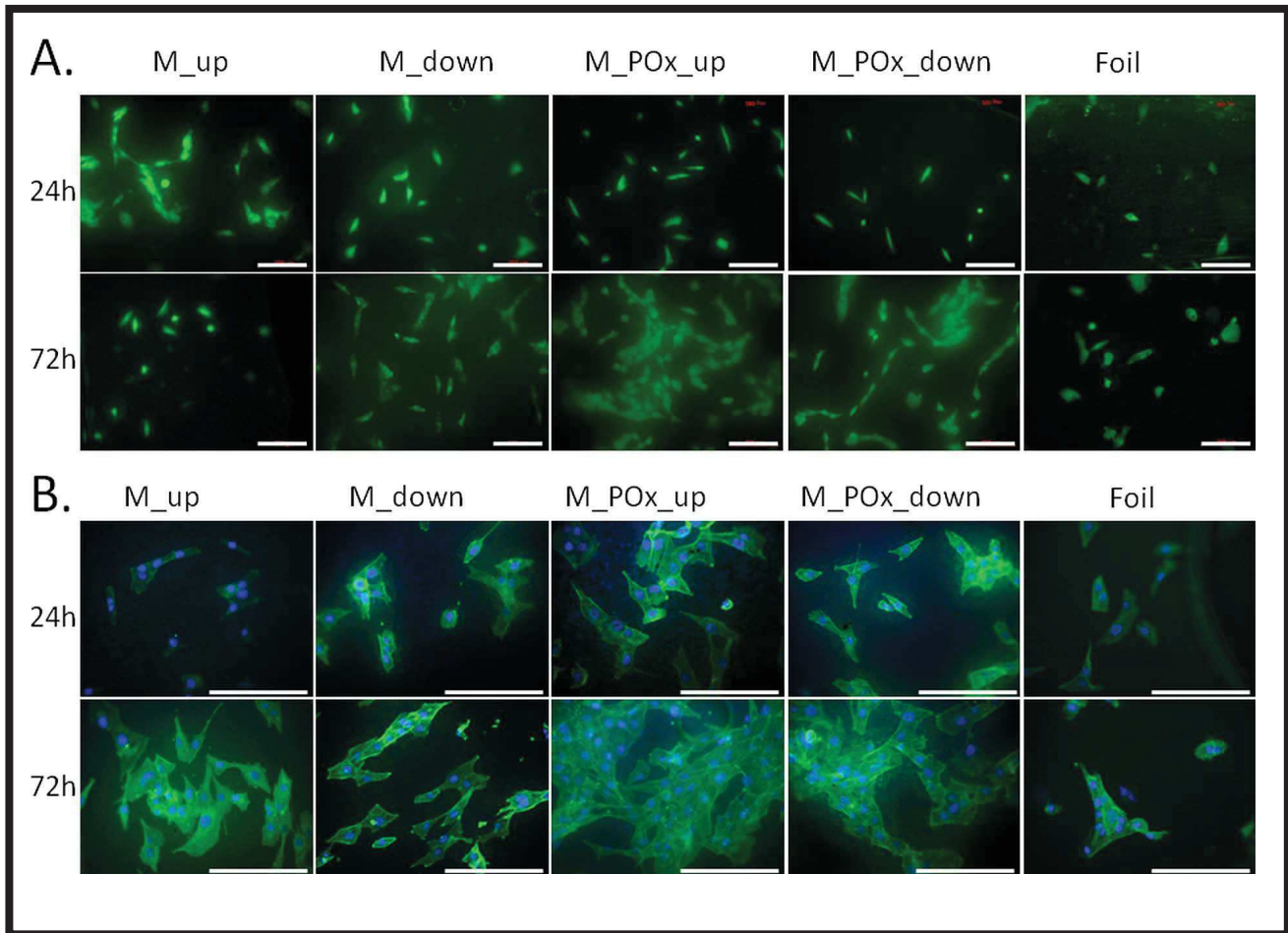


FIG. 6. Morphology of MG-63 cells cultured on PLGA membranes (M), membranes modified with POx (M_POx), and foil; up / down – surfaces in contact with air / glass during preparation. Cells stained live/dead (A) and for phalloidin/DAPI (B). Scale bar = 100 μm .

Microscopic observations of MG-63 cells after live/dead staining show that all cells on 24 h and 72 h of culture were stained green, i.e. were alive; no dead cells i.e. stained red were found (FIG. 6 A). Cells were homogenously distributed and their morphology was similar to that on PLGA foil. The number of adhering cells after 72 h looked higher on the membranes modified with POx.

Cells cultured on all membranes were better spread and had better developed actin fibres as compared to cells cultured on foils for 24 h (FIG. 6 B). After 72 h cells cultured on membranes with POx showed the most flattened morphology with intensively stained cytoskeleton.

Conclusions

In this study, we developed a method of manufacturing of porous asymmetric PLGA membranes modified with POx. The membranes were obtained by phase separation and preferential adsorption of POx molecules at the interface PLGA-POx-PEG followed by PEG leaching. Surface modification of PLGA with POx was confirmed by FTIR-ATR, by the presence of a band at about 1620 cm^{-1} , attributed to C=O and C-N stretching vibrations from poly(2-oxazolines), which was not found in the spectra of PLGA nor PEG.

Addition of POx influenced topography of the membranes, by decreasing average pore size on the glass-cured surface of the membrane and decreasing surface roughness of the air-cured surface of the membranes. Interestingly, the addition of POx did not influence membranes' wettability.

All membranes were found cytocompatible with osteoblast-like cells. Presence of POx resulted in better cell adhesion as shown by microscopic studies after fluorescence staining for nuclei and cytoskeleton actin fibers.

To sum up, one-step phase separation process between PLGA, POx, and PEG, dissolved in DCM followed by drying and PEG leaching resulted in cytocompatible, asymmetric PLGA membranes, which might be considered for guided tissue regeneration technique in periodontology and in bone tissue engineering.

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