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The use of long-chain plant polyprenols as a means to modify the biological properties of new biodegradable polyurethane scaffolds for tissue engineering. A pilot study

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Abstract Microporous membranes for tissue engineering were produced from new biodegradable polyurethane based on hexamethylene diisocyanate, poly(ε-caprolactone) diol and 1,4:3,6-dianhydro-D-sorbitol. The interconnected pores had an average size in the range of 5–100 μm. The tensile strength at break, the Young's modulus and elongation at break of the membranes were 3.2 ± 0.3 MPa, 25.2 ± 1.5 MPa and 190 ± 12%, respectively, while nonporous foils from the same polymers had a tensile strength at break of 40 ± 2 MPa, a Young's modulus of 91 ± 6 MPa, and an elongation at break of 370 ± 10%. The membranes were incubated for 10 days in a 2.65 vol% solution of long-chain plant polyprenol in *n*-hexane to promote their interaction with cells and tissues. The polyprenol was isolated from leaves of *Magnolia cobus* and was a mixture of prenol-10 and prenol-11. The prenol-impregnated membranes and nonimpregnated membranes (control) were tested in cell culture to assess whether impregnation has a beneficial effect on cell-material interaction. The cells used in the test were chondrocytes isolated from the articular-epiphyseal cartilage of leg bones of 5-day-old inbred LEW rats. The time of culture was 2 and 5 weeks. Both, the nonimpregnated and impregnated polyurethane membranes supported attachment and growth of rat chondrocytes. The cells firmly attached to the surface of the microporous membranes, invaded the pores and maintained the round shape characteristic for chondrocyte-like-morphology. Abundant fibrillar extracellular matrix produced by the cells resembled the network formed by chondrocytes in vivo. The cells produced relatively more extracellular matrix in the membranes impregnated with polyprenol than in the control membranes. Impregnation of polyurethane scaffolds with biologically active amphiphilic polyprenols may be a route to facilitate the cell–material interaction.

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Introduction

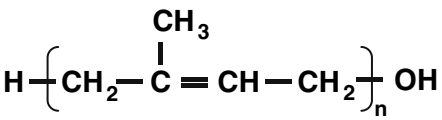
The number of patients suffering from tissue or organ failure is increasing, while the supply of autogenous tissues and organs for transplantation is limited. This calls for new modalities to treat these problems. Potentially, "artificial" tissues and organs might be used instead of autogenous ones, providing their biological functionality approximates that of the original tissues and organs. One of the routes which might lead to such tissue substitutes is tissue engineering where a construct consisting of a suitable scaffold seeded with autogenous cells is implanted in place of damaged or malfunctioning tissues and organs. An important component in reaching such a goal is the availability of a suitable scaffold for cells.

Among candidate biomaterials for scaffolds bioresorbable polymers of natural or synthetic origin and/or ceramics play an important role. The type of biomaterial

for scaffolds depends on the type of tissue to be repaired, i.e. potentially different materials may be needed for the repair of hard and soft tissues. It is generally appreciated that scaffolds for tissue engineering should support attachment, spreading and proliferation of cells. It should allow for the production and maintenance of the extracellular matrix. The scaffold should be microporous with interconnecting pores of suitable size to allow for the ingrowth of cells, blood vessels and tissues. It should be produced from biocompatible and bioresorbable/biodegradable materials to allow for the gradual replacement of the scaffold matrix with newly formed tissue. Optimally, the scaffold's resorption time should match the rate at which the new tissue is formed. The scaffold's mechanical properties should ensure its functionality.

Polyhydroxyacids are the most frequently used of the candidate bioresorbable polymers for scaffolds. Yet another group of polymers with potential application for tissue engineering are biodegradable polyurethanes. These polymers can be synthesized as hydrophilic, hydrophobic or amphiphilic depending on the intended application. Their mechanical properties and rates of degradation can be well controlled [1–16] and the interaction with cells and tissues can be modulated. This can be achieved by using biologically active compounds, e.g. growth factors, pharmacologically active substances such as amino acids or selected polysaccharides. Yet, another group of pharmacologically active substances which might potentially be used to enhance the interaction of scaffolds with cells and tissues are plant polyprenols. These compounds can be deposited in the scaffolds by impregnation, for example. The versatile chemistry of polyurethanes also allows the incorporation of polyprenols into the polyurethane backbone chain or as side chains upon synthesis.

The term prenol is a contracted name for isoprenoid alcohol with the formula [17]:



Polyprenols represent a subgroup of prenols in which *n* is greater than 4. A polyprenol amphiphylic molecule consists of a hydroxyl group (a hydrophilic part), and a long unsaturated isoprenyl chain (the repeating isoprene residues) mainly of poly-*cis* configuration (a hydrophobic part). Plant poly-*cis* prenols with the structure ωtxcyOH (where, ω is an isoprene residue farthest from the hydroxyl group, t is a *trans*-isoprene residue, c is a *cis*-isoprene residue and –OH is the hydroxyl group) contain two or three internal *trans* isoprene units [18, 19]. The structures of the polyisopolyprenol, the isoprene unit and dolichol molecules are presented in Fig. 1. Plant polyprenols are

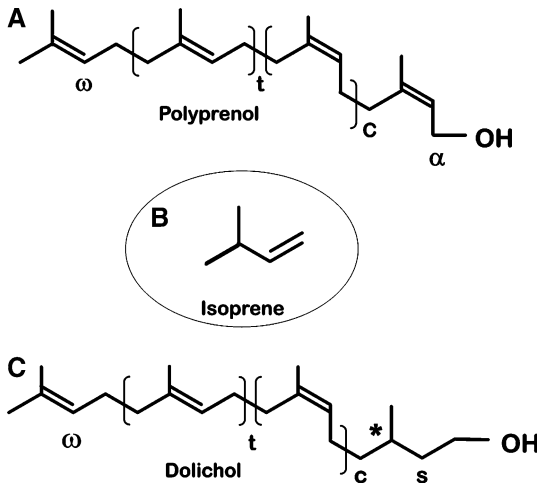


Fig. 1 The chemical structure of polyisoprenols. (**A**) Polyprenol; (**B**) Isoprene unit; (**C**) Dolichol molecule

reported to be pharmacologically active [20]. Polyprenols also seem to affect the growth of cells in culture. Alpha-saturated polyprenols induce phenotypic changes in Ehrlich ascites tumour (EAT) cells. EAT cells attach to glass and spread on it but grow in an overlapping pattern. It is suggested that polyprenols may represent a class of compounds which by interference with the biosynthesis of plasma membrane constituents influence the surface properties of EAT cells and induce spreading [21].

The purpose of this pilot study was to evaluate whether impregnation of the biodegradable polyurethane membranous porous scaffolds with a selected long-chain plant polyprenol has a beneficial effect on their interaction with chondrocytes in culture. The scaffolds were designed as an "artificial periosteum" for the repair of articular cartilage defects.

Experimental

Biodegradable polyurethane membranes

The biodegradable linear polyurethane used for the preparation of the microporous membranes was synthesized in a two-step bulk polymerization. The monomers used were aliphatic hexamethylene diisocyanate (HMDI) (Sigma, Milwaukee, USA), poly(ε-caprolactone) diol (PCL) with a molecular weight of 530 (Aldrich, Milwaukee, USA) and isosorbide diol (Iso) (1,4:3,6-dianhydro-D-sorbitol) chain extender (Aldrich, USA). Dibutyltin dilaurate (DBDL) was used as catalyst. Details of the polymer synthesis have been described elsewhere [12, 13]. The microporous membranes were prepared from the polymer solution in a mixture of dimethylsulfoxide (DMSO) and acetone using a phase-inverse process. The membranes were formed on

PTFE-coated rollers (diameter 30 mm, length of 150 mm) using water as a precipitant. Deposition on the rollers of 30 layers of the polyurethane solution provided microporous membranes with satisfactory mechanical properties, i.e. the membranes could be easily sutured. The membranes were rinsed in a mixture of water and ethanol (80:20 vol:vol/%) and subsequently dried a vacuum oven at 50°C. Circular samples with a diameter of 14 mm were cut from the membranes and incubated at room temperature for 10 days in a solution of polyprenols in hexane (2.65 vol%). The membranes after incubation were dried at 50°C to a constant weight in a vacuum oven, fixed between PTFE rings, packed in double pouches, sterilized by a cold-cycle ETO process, and then evacuated again at 50°C and 4×10^{-1} mbar for 5 h [14].

Isolation of polyprenols

Long-chain polyprenol (a mixture of prenol-10 and prenol-11) was isolated from leaves of *Magnolia cobus*. Dried leaves (200 mg) were homogenized at high speed for 1 min in acetone–hexane 1:1 v/v mixture using an Ultra-Turrax T25 mixer. Next the extract was subjected to alkaline hydrolysis [22]. Analytical separation of polyprenols was performed by TLC on Silica gel plates in ethyl acetate:toluene 5:95 v/v mixture and on RP-18 plates in acetone. Spots of lipids were detected with iodine vapour and identified with standards. The unsaponifiable lipids were chromatographed on a Silica Gel 60 column (Merck, Darmstadt, Germany) and eluted with hexane containing increasing concentration of diethyl ether (0–18%). The course of elution was monitored by TLC. A semiquantitative determination of polyprenols was performed using an adsorption chromatography by comparing the size and intensity of the detected spot with that of a known amount of a standard substance [23]. All organic solvents used for extraction and chromatography were from Merck (Darmstadt, Germany). Silica gel TLC plates and R18-plates with concentrating zone and silica gel for column chromatography were also from this source. After isolation the polyprenols produced single spots on Silica Gel G TLC plates in ethyl acetate/toluene 5:95 v/v mixture and on RP-18 HP TLC plates in acetone.

Characterization of polyurethane membranes and polyprenol

Thermal analysis

A Perkin-Elmer (Norwalk, CONN) differential scanning calorimeter (Pyris DSC-1) calibrated with indium was used to evaluate the thermal properties of the polyurethane membranes and polyprenols. The weight of the polymer samples was 5–8 mg, and polyprenol 5–6 mg. The samples were scanned at a heating rate of 10°C/min under dry, oxygen-free nitrogen flowing at a rate of 50–60 ml/min. The samples were scanned from 15°C to 120°C [12, 13].

Mechanical properties

Tensile strength and modulus of as-produced polyurethane membranes and the membranes impregnated with polyprenol were measured using an Instron tester model 4302 (High Wycombe, Bucks, England). The tester was equipped with a 0.1 kN load cell operating at a cross-head speed of 10 mm/min. The samples for tests were Type V tensile bars (ASTM D638) [12].

Infrared spectroscopy

Infrared spectra of the polyurethane, the polyprenol and the polyurethane membranes impregnated with polyprenol were recorded in transmission and reflection modes using a Fourier-Transform Perkin Elmer 2000 FT–IR spectrometer (Beaconsfield, Buckinghamshire, England). An attenuated total reflection (ATR) unit was fitted with KRS-5 crystal (45° entrance angle). About 30 scans were taken for each sample [16].

Scanning electron microscopy of polyurethane scaffolds

A Hitachi (Tokyo, Japan) model S-4100 field emission scanning electron microscope operated at 2.0 kV was used to observe the polyurethane samples sputtered with a 5 nm thick platinum layer [14].

Electron microscopy of chondrocytes on polyurethane scaffolds

A JEOL JEM1200EX transmission electron microscope with a scanning attachment unit (Tokyo, Japan) operated at an accelerating voltage of 80 kV was used to observe chondrocytes on polyurethane scaffolds. Samples were sputtered with gold layer.

Chondrocyte isolation

Under aseptic conditions the articular-epiphyseal cartilage fragments were collected from the leg bones of 5-day-old inbred LEW rats and digested in 0.25% collagenase, 0.05% DNase and tosyl-L-lysine chloromethyl ketone (TLCK) for 36 h. The chondrocytes released after filtration were centrifuged at 200–300 G for 7 min and then seeded onto scaffolds kept in a 24-well plate. There were 250,000 cells

in 1 ml of medium seeded onto each scaffold. The cells were cultured at 37°C for 2 and 5 weeks in DMEM-F12 containing 10% calf serum (50 μg/ml) and antibiotics (Penicillin 10000 IU/ml, Streptomycin 10000 μg/ml and Aphotericin B 25 μg/ml) (ICN Biomedicals, Inc).

Cell morphology

At the end of the experimental period, the scaffolds with cells were rinsed three times with PBS and were then fixed in a mixture of 2.5% gluteraldehyde, postfixed in 1% osmium teroxide, dehydrated for 10 min in ethanol with concentrations of 50, 70, 80, 90, 96 and 100% and additionally for 30 min in 100% ethanol. Next, the samples were dried in a critical point dryer, sputtered with gold layer and observed under the transmission electron microscope.

Results and discussion

Microporous polyurethane membranes

A scanning electron microscopy image of the surface of the microporous elastomeric polyurethane membranes which were seeded with cells is shown in Fig. 2. The membrane had interconnected pores with an average size in the range of 5–100 μm. The tensile strength at break, the Young's modulus and the elongation at break of the microporous membranes were 3.2 ± 0.3 MPa, 25.2 ± 1.5 MPa and 190 ± 12%, respectively. The membranes could be easily handled and sutured. The nonporous foils from the same polymers had a tensile strength at break of 40 ± 2 MPa, a Young's modulus of 91 ± 6 MPa, and an elongation at break of 370 ± 10% [12].

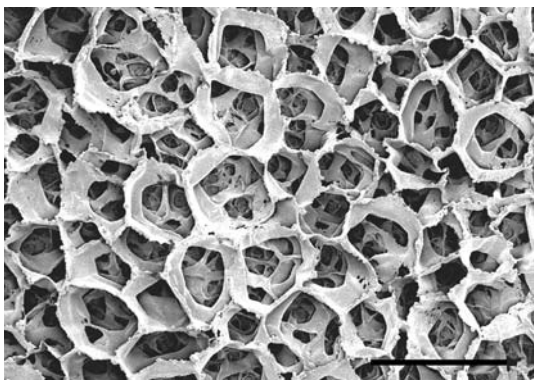


Fig. 2 Scanning electron microscopy image of the surface of the microporous polyurethane membranes seeded with cells. Scale bar represents 100 μm

Thermal characteristics of polyprenol mixture

The DSC thermogram of the mixture of long-chain polyprenols (decaprenol and undecaprenol) is shown in Fig. 3. The thermogram shows two thermal transitions at temperatures in the range of 35.5–39.7°C. The melting peak temperature at 36.4°C also called pretransition corresponds to the crystal–crystal transition. The high-energy melting endotherm at 38.0°C with $\Delta H = 3.55$ J/g corresponds to the gel-to-liquid crystal transition or the solid-to-mesophase transition [24, 25]. The thermal transitions of the polyprenols used in the study were similar to the thermal transitions of distearoyl phosphatidylcholin (DSPC), dipalmitoyl phosphatidylcholin (DPPC) and dimyristoyl phosphatidylcholin (DMPC) lipids which are present in cell membranes. Transition temperatures of these lipids were 23, 42 and 54°C, respectively [26].

Infrared spectroscopy

Typical infrared spectra of the polyurethane, the polyprenol and the polyurethane membrane impregnated with polyprenol used in the study are shown in Fig. 4. The spectrum of the polyurethane shows characteristic IR bands which were described in detail previously [9]. The peak at 1681 and 1533 cm^{-1} are typical for ν(C=O) (amide I) and δ(NH) with ν(CO–N) (amide II) while the peak at 1724 cm^{-1} is typical for the ester ν(C=O) present in the polyurethane [9].

In the infrared spectrum of the polyprenol there were absorption bands at 2962, 1449 and 1376 cm^{-1} which were assigned to the methylene $-CH_3$ groups and the weak peaks in the range of 1667–1580 cm^{-1} assigned to the –C=C– group. Both groups are specific for the polyprenol. The infrared spectrum of polyurethane membranes impregnated with polyprenols shows one absorption band at 1376 cm^{-1} assigned to the methylene group originating exclusively from polyprenols. The presence of this adsorption band

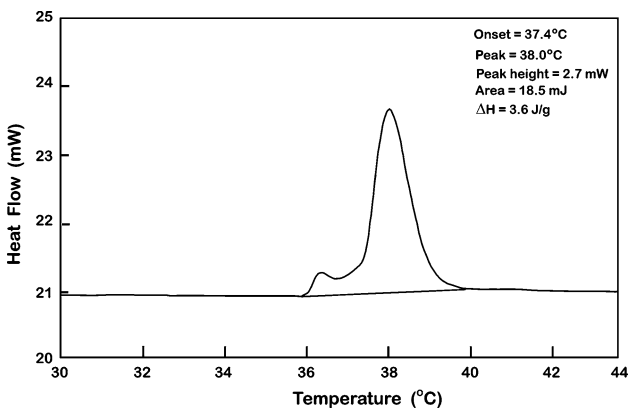


Fig. 3 DSC thermogram of the polyprenol samples

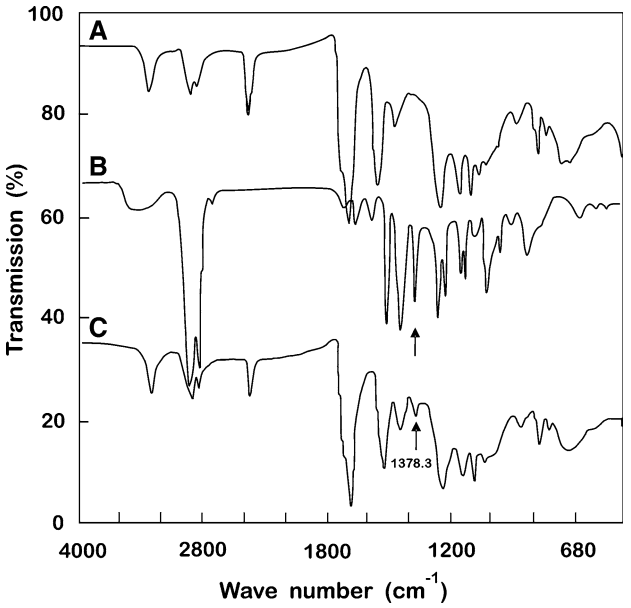


Fig. 4 Infrared spectra of the materials used in the study. (**A**) Polyurethane membrane; (**B**) Polyprenols; (**C**) Polyurethane membranes impregnated with polyprenols

confirmed the successful impregnation of polymeric membranes with polyprenols. The other adsorption bands found in the spectra of the polyprenols are not present in the spectra of the polyurethane scaffolds impregnated with polyprenols. These bands are masked by strong adsorption bands originating from the chemical groups of the polyurethane.

Cell growth on the polyurethane membranes

Throughout the whole culture period of 5 weeks both, the nonimpregnated an impregnated polyurethane membranes supported attachment and growth of rat chondrocytes. The cells firmly attached to the surface of the microporous membranes, invaded the pores and maintained the round shape characteristic for chondrocyte-like-morphology. During the first 2 weeks of culturing the number of cells was comparable for nonmodified and modified membranes. The cells attached firmly to the membrane surface, grew deeply into the pores and deposited fibrillar extracellular matrix. Chondrocytes growing into the pores of the nonmodified membrane at 2 weeks are shown in Fig. 5. The cells with diameters of about 10 μm maintained a round shape. The surface of the cells was rough with grainy texture. The chondrocytes growing into the porous membranes modified with long-chain polyprenols at 2 weeks are shown in Fig. 6. Chondrocytes also maintained a spherical shape in this case. Frequently, a few cells invaded the same pore. In Fig. 6 chondrocytes with diameters of approximately 5 μm are in direct contact with pore walls.

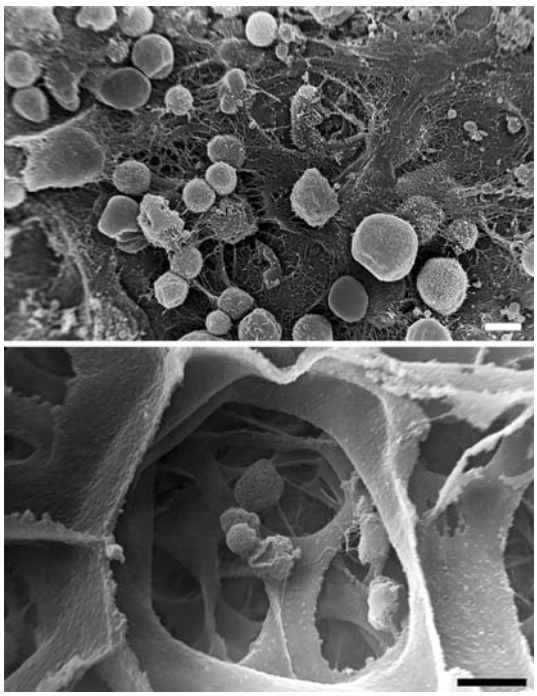


Fig. 5 Scanning electron micrographs of rat chondrocytes growing on the polyurethane membrane not modified with polyprenols at 2 weeks of cell culture. Scale bars represent 10 μm

The chondrocytes shown in Fig. 6 are in contact with the surface of the pores via abundant fibrillar matrix. During subsequent weeks of culturing the number of chondrocytes

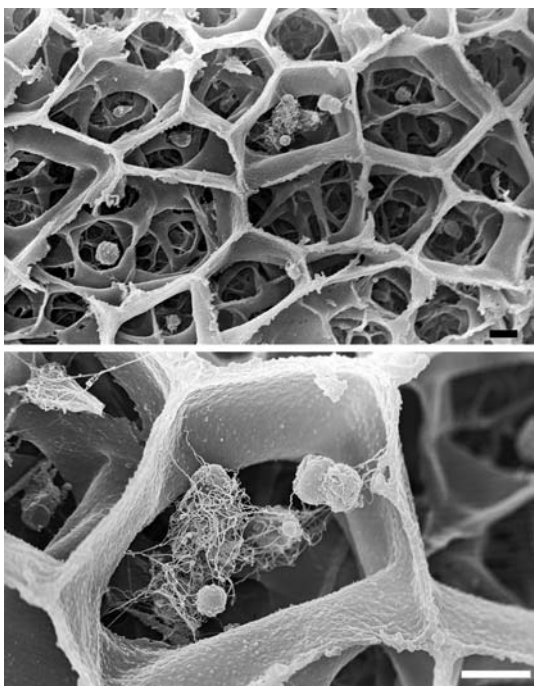


Fig. 6 Scanning electron micrographs of rat chondrocytes growing on the polyurethane membranes modified with polyprenols at 2 weeks of cell culture. Scale bars represent 10 μm

increased, in both nonmodified membranes and membranes modified with prenols. The cells produced a three-dimensional fibrillar network. The morphology of a chondrocyte with a diameter of about 15 μm growing on the nonmodified membrane at 5 weeks is shown in Fig. 7. The cell is attached to the membrane surface via a fibrillar matrix. At 5 weeks there were a large number of chondrocytes in the membrane modified with polyprenols (Fig. 8). The morphology of cells communicating with each other and firmly attached to the pore walls via podia and fibrillar extracellular matrix is shown in Fig. 8.

Loading drugs into scaffolds for tissue engineering by physical means, e.g. by impregnation always rises a question of how stable is the coating? In a separate study the authors addressed the questions of the stability of coating and the kinetics of release of polyprenols from scaffolds produced from the same polyurethane but having various geometries (membranes and sponges).

Summary and conclusions

The microporous membranes from new biodegradable polyurethanes used in the study support attachment and growth of rat chondrocytes. The cells invaded the pores of the membranes, maintained round morphology and produced abundant fibrillar extracellular matrix resembling the network formed by chondrocytes in vivo. Impregnation of the membranes with biologically active amphiphylic polyprenol with the poly-*cis* configuration of the isoprenoid chain seems to facilitate the cell–material interaction. Further extensive quantitative studies are required, however, to verify the extent to which this interaction is affected by the presence of the polyprenols in the scaffold.

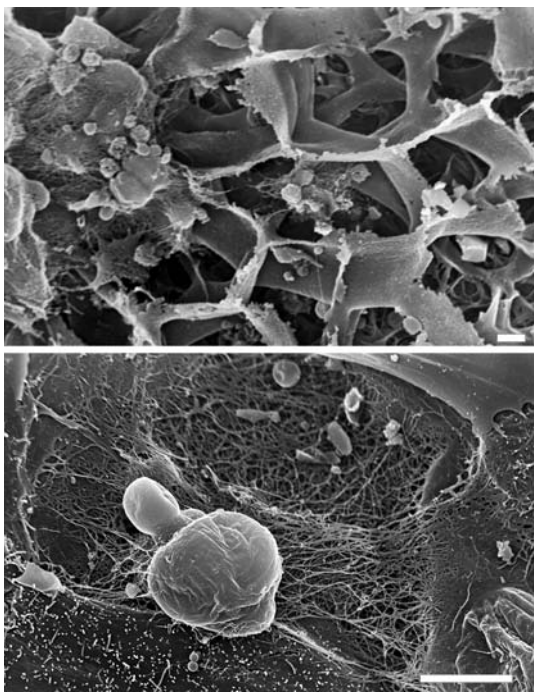


Fig. 7 Scanning electron micrographs of rat chondrocytes growing on the polyurethane membranes not modified with polyprenols at 5 weeks of cell culture. Scale bars represent 10 μm

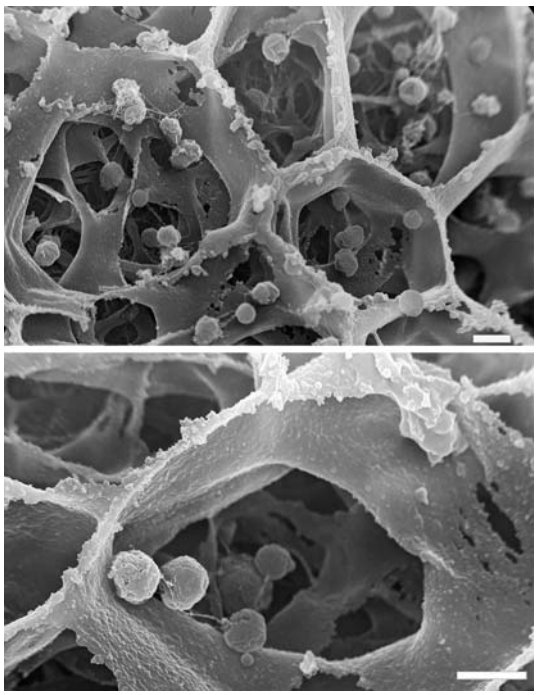


Fig. 8 Scanning electron micrographs of rat chondrocytes growing on the polyurethane membranes modified with polyprenols at 5 weeks of cell culture. Scale bars represent 10 μm

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