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Poly(HEMA) hydrogels with controlled pore architecture for tissue regeneration applications

Hana Studenovská · Miroslav Šlouf · František Rypáček

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Abstract The technique for fabrication of soft porous hydrogels, in which both the size and the orientation of inner pores can be controlled, was developed. Three-dimensional hydrophilic gels based on poly[2-hydroxyethyl methacrylate] are designed as scaffolds for regeneration of soft tissues, e.g., nerve tissue. Anisotropic macropores of the size ranging from 10 to 50 μm were formed (1) by using a porogen-leaching method with a solid organic porogen, (2) by phase-separation during gelation in solvent-nonsolvent mixture, or (3) by combination of solid porogen elimination and phase-separation. As a porogen, poly(L-lactide) fibers were applied and consequently washed away under mild conditions to obtain desired spatial orientation of pores. Highly water-swollen polymer gels were characterized with high pressure (low vacuum) scanning electron microscopy (AquaSEM). The morphology of voids remaining after removing the solid PLLA porogen (the macropores) was clearly shown.

Introduction

The interest in novel materials that could support stem cells in guided tissue regeneration increases with growing knowledge of stem cell biology. It has been found that in order for the cells to maintain or develop their tissue-specific functions they should be implanted together with a substrate material-scaffold, typically a three-dimensional supporting structure. Synthetic biodegradable polymers are attractive candidates for fabrication of tissue engineering scaffolds because they do not carry the risk of pathogen transmission and immunorejection. Each tissue has its characteristic organization, which is closely related to its physiological function, and requires that a 3-D structure of the scaffold can be adjustable accordingly.

Many tissues, including those of peripheral (PNS) and central nervous systems (CNS) are capable of healing and regeneration. Neural tissue, due to its complex and long-distance interconnections, must heal by true regeneration, because healing by scar will not reestablish the connectivity of neural signal [1]. In both nervous systems (PNS, CNS), the regeneration is limited by the fact that adult neurons are not capable of proliferation. By combination of stem cells preparation and new biomaterials for nerve guides and scaffolds, new possibilities for cell therapy in neural system are being opened.

Based on the outcomes of studies with the materials already used and explored, the requirements for a more advanced material can be defined. Hydrogels, polymer-based networks swollen in water, should have viscoelastic behavior tuned to be similar to that of soft biological tissues. In general, neurite extension on 2-D surface was usually better than in case of cells embedded in 3-D continuous substrates. A 3-D scaffold structure with abundance of interconnected, macroscopic, permanent pores should provide for required surface area of 2-D surfaces organized in a 3-D manner. Macroporous hydrogels with partially oriented pore structure or with a capacity for defined pore architecture could be suitable materials. Anisotropic distribution of pores in a hydrogel scaffold can influence the progress of nerve regeneration through different growth cones response with respect to morphology features (orientation of pores)

H. Studenovská (✉) · M. Šlouf · F. Rypáček
Department of Bioanalogous and Special Polymers, Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovsky Sq. 2, 162 06 Prague 6, Czech Republic
e-mail: studenovska@imc.cas.cz

or biochemical gradients (cytokines, growth, and trophic factors) [2, 3].

In addition, the hydrogel should have capacity to incorporate some components of extracellular matrix (ECM), such as collagen, laminin, hyaluronic acid, and fibronectin [4, 5], or expose on surfaces of its pores peptide sequences derived from the ECM proteins, e.g., RGD sequence of fibronectin [6–8].

We work on preparation of a soft hydrogel material that would allow for design of complex pore morphology, e.g., such that might be suitable in regeneration of neural tissues. In addition to appropriate mechanical properties, matching those of surrounding tissues, extensive three-dimensional connectivity between pores of the scaffold material is sought. This contribution is focused on development of a method that would provide for controlled porosity and pore architecture in soft hydrophilic methacrylate gels. As a model hydrogel for the study poly[2-hydroxyethyl methacrylate] (polyHEMA) gels were chosen [9]. In this work, macroporosity in gels was formed by using (a) a porogen-leaching method with a solid organic porogen, (b) a phase-separation method in a polymer solvent-nonsolvent mixtures, and (c) a combination of solid porogen and solvent-nonsolvent systems. PolyHEMA gels are polymers of well-established biological inertness, chemical stability, good biocompatibility, and low level of immunogenicity even if implanted into neural tissue [10–12]. However the methacrylate gel is covalently crosslinked and nondegradable, it fulfills well the role of a model for development of technique for porous scaffold formation and can provide 3-D structures, on which the significance of spatially designed pore architecture can be tested. The application of conditions developed here for polyHEMA to more complex degradable systems, such as degradable polypeptide-based hydrogels, is in progress.

Materials and methods

2-hydroxyethyl methacrylate (HEMA, Röhm GmbH, Germany) and ethylene dimethacrylate (EDMA, Ugilor S.A., France) were purified by distillation. 2,2′-azobisisobutyronitrile (AIBN, Fluka) was crystallized from ethanol. 2-aminoethanol and polyethylene glycol, PEG (M_w = 300) were purchased from Fluka, other solvents and reagents were obtained from Lach-Ner LtD and used without purification. Melt-spun poly(L-lactide) (PLLA) fibers of average diameter 12 ± 1 μm were a kind gift by Dr. J-F. Selin, of Neste Co., Finland. Poly(DL-lactide), PDLLA (M_w = 360,000), was synthesized according to procedure published elsewhere [13].

Preparation of gels

Gel samples were prepared in a form of cylinders using a mold made of a silicone tube (inner diameter = 2 mm, outer diameter = 5.5 mm). The silicon mold was first swollen in cyclohexane and the desired amount of the organic porogen, PLLA fibers, was filled in the mold. After evaporation of swelling solvent the mold shrunk to its original size. The content of porogen was determined by weighing and was varied in the range of 0.140–0.796 g/cm^3. The volume fraction of porogen was determined from the known volume of the mold and the weight of the contained porogen using ρ = 1.27 g/cm^3 for density of PLLA [14]. The mold with porogen was purged with nitrogen before filling with a polymerization mixture.

Crosslinked hydrogels were prepared by radical polymerization of HEMA-EDMA solutions, using AIBN as initiator. In some experiments, the polymerization mixtures contained glycerol/ethanol or PEG/water as a diluent (5–50% v/v) and/or water as a precipitant (at least 80% v/v). The content of crosslinker (EDMA) varied in a range of 0.4–5.0% (w/w) of the total monomers in the feed. The polymerization feed composition of individual samples is given in Table 1. The monomer mixture was purged with nitrogen and frozen/thawn in liquid nitrogen followed by application of vacuum three times to remove oxygen from the mixture. During polymerization the molds were immersed in a disulfite bath at 70 °C for 16 h.

Elimination of porogen

Two methods for porogen fibers degradation and elimination were tested and compared. In the first method, the polyester porogen was removed by hydrolysis in a sodium hydroxide solution (concentration of 1–3% w/v) at 70 °C for 10 h, followed by extraction with methanol and chloroform for 4 days. In the second procedure, the aminolysis of PLLA fibers was carried out in dichloromethane/tetrahydrofurane mixture (DCM/THF 50/50 v/v) with 2-aminoethanol (AE) (20 mL of AE/1,000 mL of DCM/THF mixture) at 50 °C for 24 h. The gel was washed with DCM/THF mixture, acetone, acetone/water mixtures, and excessively with water, to secure complete removal of any organic solvent or reactant residues. In both procedures, the gels were finally swollen to equilibrium in water.

Modification of porogen fibers

The effect of pre-coating of PLLA fibers with PDLLA on hydrogel morphology was studied. PLLA fibers were loaded in the silicon mold according to procedure described above. The mold with fibers was flushed with a solution of PDLLA in acetone (1–5% w/v), the excess of

Table 1 The polymerization feed composition (volume fraction) and other conditions of hydrogel samples preparation

Sample (Figure No.)	HEMA (%)	EDMA (%)	Diluent (%)			Precipitant (%)	LC_{PLLA} (g/cm^3)	Elimination of porogen	Fiber pre-coating
			G/E	PEG	W				
2a	70.6	0.4	29	–	–	–	0.159	NaOH	–
2b	70.6	0.4	29	–	–	–	0.159	AE	–
3a, 3b	52.6	0.4	–	28	19	–	0.796	AE	–
4a	10.0	5.0	–	5	–	80	0.287	AE	–
4b	5.0	5.0	–	–	–	90	0.092	AE	–
4c	5.0	5.0	–	20	70	–	0.092	AE	–
5a	52.6	0.4	–	28	19	–	0.790	AE	1% PDLLA
5b	52.6	0.4	–	28	19	–	0.790	AE	2% PDLLA
5c	15.0	5.0	–	–	–	80	0.796	AE	5% PDLLA

HEMA: 2-hydroxyethyl methacrylate; EDMA: ethylene dimethacrylate; G/E: glycerol/ethanol (50/50 v/v); PEG: polyethylene glycol; W: water; LC_{PLLA}: loading content of porogen; AE: 2-aminoethanol

solution was removed under mild vacuum and residual solvent was removed by drying in oven at 35°C for 20 h.

Swelling properties of gels

Samples of hydrogels were swollen to equilibrium in water, at least for 5 days. The water content in the gels and the equilibrium-swelling ratio were determined by weighing. The swollen samples weighed at equilibrium (W_s) were then dried at ambient temperature in vacuum desiccators over phosphorous pentoxide to constant weight, thus obtaining the weight of dry gel (W_d). The equilibrium water content in the swollen gel was obtained as $w_T = (W_s–W_d)/W_d$. The total volume of water (w_T) is composed of the water contained within the macropores, i.e., voids created by removing the solid porogen and the water within the swollen gel matrix. For homogeneous gels (polymerized without a precipitant) the volume fraction of water-filled pores can be estimated from the volume fraction of porogen at the gel formation. The volume fraction of the porogen was obtained from its weight fraction and density (ρ_{PLLA} = 1.27 g/cm^3).

Morphology of porous polymer gels

The morphology of the gel, as it could exist in the swollen state in aqueous environments of body fluids, was observed by a high-pressure (low-vacuum) scanning electron microscopy AquaSEM (TESCAN, Czech Republic). The pressure in the AquaSEM microscope chamber can be as high as 500–1100 Pa, which makes it possible to observe samples still containing frozen water.

The gel samples, typically of cylindrical shape (about 1 cm long and 2 mm in diameter), were kept in distilled water. Thin slab specimens (<0.5 mm thick), either cross- or tangential-sections, were cut from different parts of each cylinder, and instantly frozen in liquid nitrogen. The frozen specimen was placed on a cooled sample stage (–20 °C) of AquaSEM microscope, where it was fixed with a tiny amount of water (the water freezes immediately in contact with the cooled sample stage). The samples were observed using two backscattered electron detectors in combination with an ionization detector. Most micrographs were taken after all the visible ice from the cut surface sublimed. On some micrographs, however, blocks of ice could be still visible.

Results and discussion

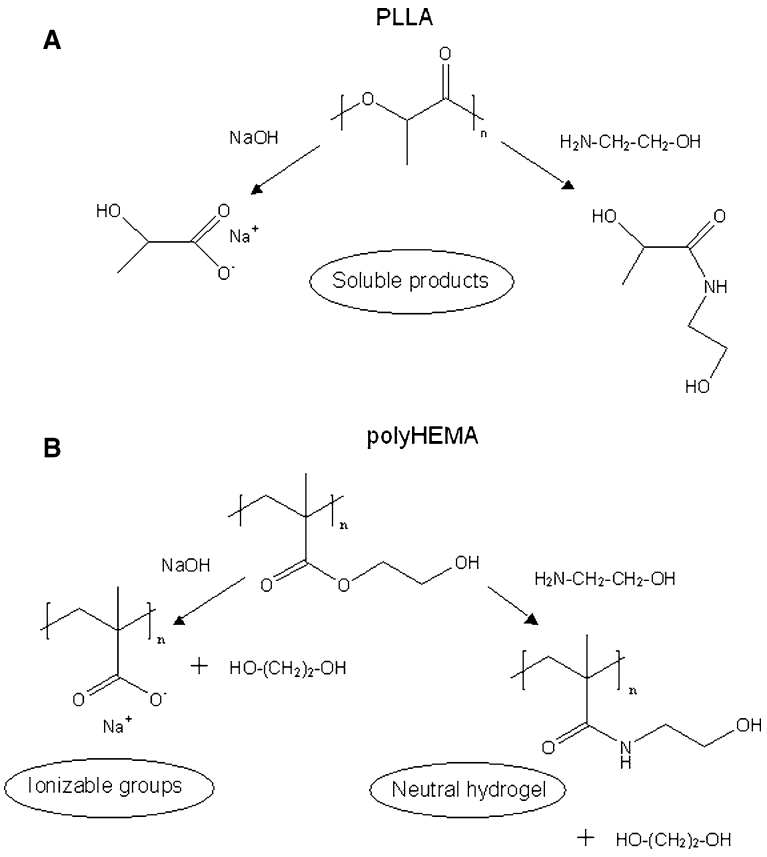
The study was carried out to explore the feasibility of preparation of hydrogel scaffolds with a high volume fraction of porosity within a soft, high-water-content hydrogel matrix. An organic porogen, which can be preformed into a defined 3-D structure, was selected and we investigated how the structure of porogen translates into resulting morphology of voids—macropores, in the hydrogel matrix. It is important to reveal the pore morphology in the gel as it could be present in the swollen state of the gel, i.e., the state in which the material would exist in the biological fluids or in the living tissues. In contrast to samples of biological materials and tissues, the structure of which, thanks to high content of proteins, can be well preserved by a treatment with chemical fixation agents commonly used in histology (e.g., formaldehyde, glutaraldehyde, etc.), the most of synthetic polymer gels cannot be efficiently fixed by these agents. In addition, unlike biological tissues, synthetic polymers often swell or even dissolve in organic solvents (e.g., ethanol, acetone, xylene, carbon dioxide) which have to be used in usual sample processing techniques for classical SEM, in order to dehydrate the sample for high vacuum, thus leading to

volume changes and, consequently, to artifacts in gel structure observed by SEM.

Therefore, another objective of the work was to test the suitability of high-pressure (low vacuum) scanning electron microscopy (AquaSEM), a technique already established for observation of wet and native biological samples, for morphological studies of swollen gels. The 3-D structure of swollen gel was quenched by rapid freezing in liquid nitrogen. Small dimensions of specimen enabled very rapid freezing, which minimizes the crystallization of water inside macropores and gel matrix. The morphology of the gel could be observed after partial sublimation of the ice, without melting or significant recrystallization of the frozen water.

AquaSEM revealed the morphology of macropores, which were created as voids remaining after removing the solid porogen, such as PLLA. Using a polymer material as a porogen gives us the option that such porogen can be preprocessed and shaped in a suitable 3-D structure before it becomes embedded in the gel matrix and thus the future pore architecture can be preformed. In our work we used PLLA fibers as a model, which make it possible to create anisotropic porosity with certain prevalent pore orientation, simply by orientation of fibers in the mold. Actually, the pores created in this way form continuous tubular channels that might, if needed, secure fluid connectivity even between the opposite surfaces of a macroscopic sample, whatever might be the sample size. Obviously, one can perceive that besides simple fibers, a more complex architecture of pores could be designed using more sophisticated techniques of polymer porogen shaping, for instance, rapid prototyping methods [15].

PLLA, a member of aliphatic polyesters family, was chosen because of its easy degradability that facilitates removal of porogen without destroying the gel structure. Two techniques for porogen elimination, hydrolysis and aminolysis, were tested and their suitability for preserving hydrogel morphology considered. The chemical reactions for hydrolysis and aminolysis processes are shown in Scheme 1. Although both processes degrade PLLA rapidly enough, they differ in their effect on the methacrylate gel matrix. During degradation of PLLA by hydrolysis a concurrent hydrolysis of some hydroxyethyl methacrylate esters may occur, which would create also some ionizable methacrylic acid side chains on methacrylate backbone of gel matrix. On the other hand, if there were any aminolysis of methacrylate ester by 2-aminoethanol, the product of this reaction would be again a neutral hydroxyethyl side chain, which does not change the swelling behavior of the gel.



Scheme 1 Scheme of degradation processes during elimination of polyester porogen. (**A**) Degradation of PLLA fibers by hydrolysis or aminolysis and (**B**) possible side reaction during treatment on polyHEMA hydrogel

The presence of ionizable groups in the gel matrix increases the gel swelling. The effect of hydrolysis in two different sodium hydroxide concentrations on the swelling behavior of polyHEMA gels is illustrated in Fig. 1. The data shows that the gels treated at more severe hydrolysis conditions, i.e., at higher sodium hydroxide concentration, exhibit higher equilibrium water content, which can be explained by the higher amount of ionizable groups in the gel matrix. The degree of swelling increases slightly with the increasing dilution during gel formation, as the cross-linking efficiency decreases with polymer dilution. For the diluent contents above 40% v/v the swelling dependence is anomalous. This behavior reflects the presence of voids (macroscopic pores) in the gel. The total content of water in the gel is composed of two contributions, the water in the swollen gel matrix and that in the water-filled macropores. When a diluent is present during the gel formation the gel is formed already in a swollen state. For high content of diluent (>40%), the gel-swelling ratio is already above its equilibrium value for swelling in water (cf. equilibrium swelling of polyHEMA in water is about 40% [16]). Therefore, when put in water, the gel matrix actually deswells to its equilibrium and expels some water from voids left after porogen, the volume of which during the gel formation is independent of the polymerization feed composition. The decrease of water fraction in the pores then accounts for the decrease of total water content determined by weighing of the swollen gel samples.

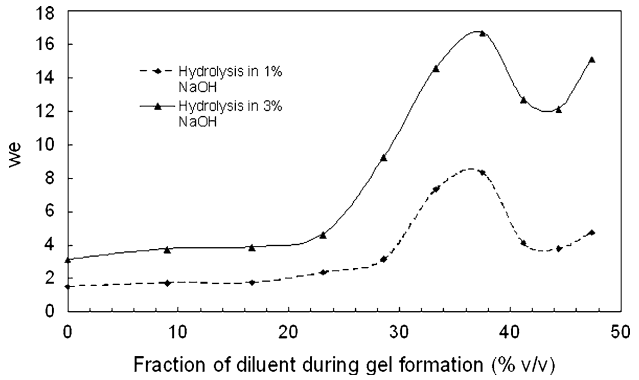


Fig. 1 Dependence of equilibrium water content on the fraction of diluent (glycerol/ethanol) during gel formation at the hydrogels with constant content of porogen (0.140 g/cm^3)

In the gel morphology, the volume fraction of pores can be controlled by the amount of porogen added. The diameter and geometry of the pores should follow the diameter and geometry of the fibrous porogen. In our samples, fibers with average diameter of 12 ± 1 μm were used.

The Fig. 2A and B compare a typical appearance of gel morphology of the gel sample polymerized under the same conditions, however, in which the porogen was eliminated by hydrolytic and aminolytic processes, respectively. The gel obtained after aminolysis of PLLA shows a regular shape of pores, with their diameter well corresponding to that of the fibers used, surrounded by a homogenous gel matrix. There are no residues of porogen remaining in the matrix. The hydrolyzed gel sample shows slightly enlarged pore dimension, due to higher swelling, and typically, also a structured gel matrix appeared. The cellular structure of the gel matrix, which appeared typically in gels with very high water content, could be interpreted as artifacts, such as imprints of crystals of water formed on the surface and, perhaps, in the gel matrix, that was highly swollen due to presence of ionizable groups. Based on these observations, the aminolytic process for elimination of polyester porogen was considered as more suitable and it was used in all other gel samples presented hereafter. It is worth noticing that the dimensions, shape and orientation of pores observed by the AquaSEM techniques corresponded very well with the dimensions and shapes of the porogen used, indicating that the observed structure really reflects the state of the swollen gel. This makes it possible to use this technique to follow more complex pore morphologies as well.

Figure 3A and B demonstrate the feasibility of creating anisotropic porosity using fibers in different prevalent orientation with respect to sample aspects.

In addition to the effect of solid porogen, which is used here to create a system of continuous channel-like pores, the porous structure of the gel can be further modified by phase separation of polymer in the presence of a polymer

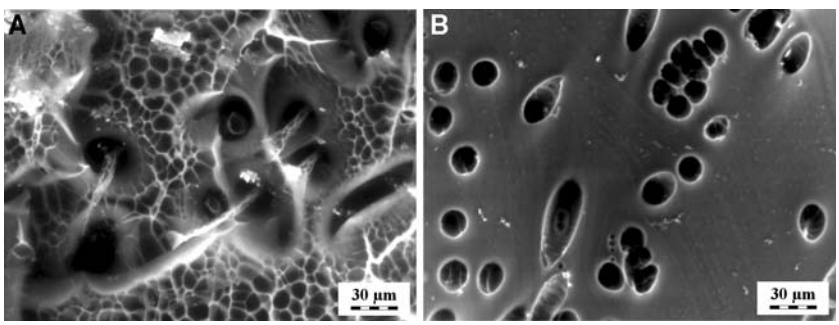


Fig. 2 Influence of different treatment for porogen elimination on polyHEMA hydrogel morphology (the polymerization feed composition of hydrogel samples is depicted in Table 1). (**A**) Hydrolysis in NaOH solution (3% w/v); (**B**) Aminolysis

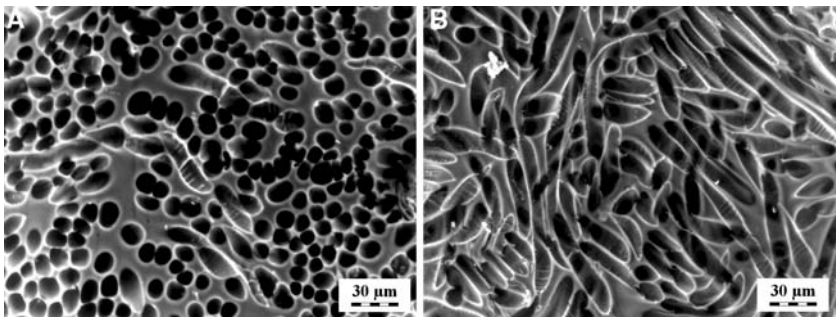


Fig. 3 Orientation of pores in polyHEMA hydrogels (the polymerization feed composition of hydrogel samples is depicted in Table 1). (**A**) Perpendicular direction of pores through the hydrogel sample; (**B**) Diagonal direction of pores through the hydrogel sample

precipitant during the gel polymerization. Since HEMA monomer is well miscible with water, which, in turn is a precipitant for polyHEMA, water was used as precipitant to introduce the phase-separation factor. In polymerizations of monomer mixtures containing more than 80% (v/v) of water, water acts as a precipitant of polyHEMA formed during the polymerization. Due to phase separation of polymer-rich and nonsolvent-rich phases, a heterogeneous gel structure is formed in the spaces between the networks of the solid porogen. In this way additional pores are formed, that interconnect the channels created by leaching the solid porogen (Fig. 4A). Thus two interpenetrating and interconnected systems of macropores are formed, one-rather isotropic and random-formed due to phase separation of the crosslinked polymer in a solvent/nonsolvent system, and the other created by means of solid porogen, which, when based on organic hydrolysable fibers, can be anisotropic and oriented in a chosen direction, providing for continuous channels throughout the gel matrix. In Fig. 4B and C the effect of precipitant/diluent on the morphology of hydrogel with the same content of fibrous porogen is shown. In Fig. 4B the low content of porogen and high amount of precipitant (90% of water) caused strong phase separation of polyHEMA, preferably on the porogen surface, which tends to web-like morphology. In comparison, in the gel on Fig. 4C with the same porogen content, the presence of diluent and the water content under the limit for precipitation of polyHEMA results in more compact hydrogel morphology.

Taking advantage of different solubility of high-molecular-weight PLLA and PDLLA in acetone, the surfaces of PLLA fibers can be coated by a PDLLA layer by applying PDLLA as an acetone solution. The effect of the poly(DL-lactide) layer at the interface between PLLA fiber and the gel matrix on the resulting gel morphology was studied. PDLLA easily swells by HEMA monomer and adsorbs hydrophobic initiator, causing that HEMA polymerizes in the PDLLA layer on the surface of porogen fibers (Fig. 5A). In presence of water as a nonsolvent, polyHEMA is thus preferentially deposited on the surfaces of porogen (Fig. 5C). By varying the ratios of total monomer content with respect to the porogen fraction, thickness of PDLLA coating and the content of precipitant, various gel morphologies, ranging from rather homogenous gel matrix surrounding the porogen-formed voids through gels composed of bundles of hollow tubes to structures formed almost exclusively by thin tubular lamellas of gel matrix, formed on the former interface between the porogen and the polymerization solution can be developed (Fig. 5A–C).

The presented results showed how it is technically feasible to create a controlled architecture of inner void spaces in a soft hydrophilic gel. In 3-D scaffolds with dimensions more then few millimeters, the exchange of solutes as well as oriented cell growth become important. Therefore, two levels of internal spaces—pores were formed. While continuous channel-like pores are envisaged to be important for oriented cell migration and tissue

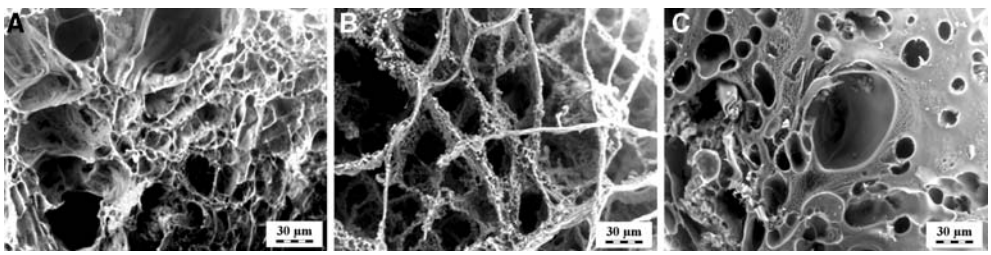


Fig. 4 Effect of phase separation on hydrogel morphology (the polymerization feed composition of hydrogel samples in details is depicted in Table 1). (**A**) Precipitant 80%, LC_{PLLA} 0.287 g/cm^3, 5% of diluent (PEG); (**B**) Precipitant 90%, LC_{PLLA} 0.092 g/cm^3, no diluent; (**C**) No precipitant, LC_{PLLA} 0.092 g/cm^3, diluent (PEG 20%, W 70%)

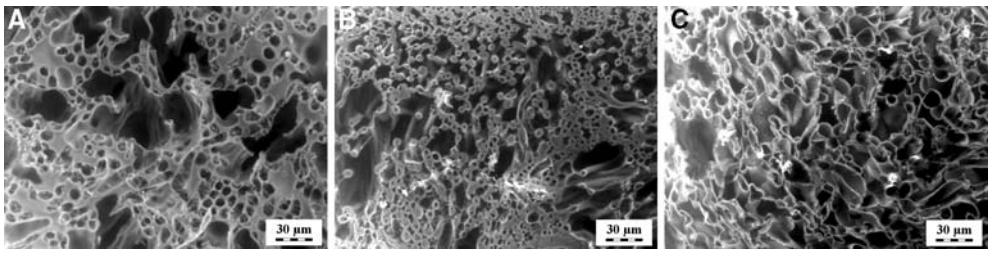


Fig. 5 Effect of pre-coating of porogen by PDLLA acetone solution on polyHEMA hydrogel morphology. (**A**) LC_{PLLA} 0.790 g/cm^3, EDMA 0.4%, pre-coating in 1% PDLLA, 47% of diluent (PEG/W); (**B**) LC_{PLLA} 0.790 g/cm^3, EDMA 0.4%, pre-coating in 2% PDLLA, 47% of diluent (PEG/W); (**C**) LC_{PLLA} 0.796 g/cm^3, EDMA 5%, pre-coating in 5% PDLLA, 80% of precipitant (W)

growth, the randomly oriented cellular pores formed by phase separation are essential for exchange of nutrients and growth factors between the channels.

Cell culture studies on the gels prepared by the described technique are in progress. Preliminary data show good biocompatibility of gels in cell cultures, thus confirming complete removal of potentially toxic solvents and/or reagents during gel preparation and washing.

Conclusions

Soft porous hydrogels based on polyHEMA were prepared. The techniques described above allow good control over the porosity, offering also the possibility to set the pore architecture and orientation. Hydrogel porosity ranging from 10 to 50 μm was detected by new technique of SEM allowing characterizing the pore structure of hydrated samples.

The polyHEMA hydrogel scaffolds described above possess key characteristics useful for tissue engineering. Studied scaffolds made by porogen-leaching technique form only one part of research. For hydrogel scaffolds use in clinical practice some other aspects like biodegradability of scaffold, presence of supporting compounds such as proteins, peptides, growth factor and other trophic factors should be considered.

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