



Synthesis of 2-Methacryloyloxyethyl Phosphorylcholine (MPC) Based P(2-hydroxyethyl methacrylate) P(HEMA) Cryogel Membranes

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Abstract

Synthesis of artificial/natural polymeric biomaterials having resistance to nonspecific protein adsorption, blood coagulation, and bacterial adhesion has attracted great attention, so nonspecific adsorption of proteins and biomolecules causes unfavorable biological responses including blood clotting, inflammation, cell adhesion, cell differentiation, and biofilm formation. A zwitterionic phosphorylcholine (PC) group of 2-methacryloyloxyethyl phosphorylcholine (MPC) is employed for biologically inert functions, especially in resistance to protein adsorption. So, it is aimed to develop bio-inspired, efficient and environmentally friendly MPC containing cryogel membranes for polymeric scaffolds for promoting cell-biomaterial. Cryogel membranes were synthesized in a semi-frozen medium by free radical polymerization in an ice bath and characterized by SEM/EDX, micro-CT, and swelling ratio measurements. In vitro biocompatibility was assessed from cell viability studies performed using cultured fibroblast cells.

Keywords: 2-Methacryloyloxyethyl Phosphorylcholine; 2-Hydroxyethyl Methacrylate; Cryogel.



2-Metakriloksietil Fosforilkolin (MPC) Temelli P(2-Hidroksietil metakrilat) P(HEMA) Kriyojel Membranların Sentezi

Öz

Spesifik olmayan protein adsorpsiyonu, kan pıhtılaşması ve bakteriyel yapışmaya dirençli yapay / doğal polimerik biyomalzemelerin sentezi büyük ilgi görmektedir, çünkü proteinlerin ve biyomoleküllerin spesifik olmayan adsorpsiyonu, kan pıhtılaşması, enflamasyon, biyofilm oluşumu, hücre yapışması ve hücre farklılaşması gibi olumsuz biyolojik tepkilere yol açmaktadır. 2-metakriloksietil fosforilkolindeki (MPC) zwitter iyonik grup olan fosforilkolin (PC) biyolojik inert fonksiyonlardan sorumludur, özellikle de özellikle protein adsorpsiyonuna direnç göstermektedir. Böylelikle, hücre-biyomateryal etkileşimlerini teşvik etmek için polimerik yapı iskeleleri olarak biyo-esinlenilmiş, verimli ve çevre dostu MPC içeren kriyojel membranların geliştirilmesi amaçlanmıştır. Kriyojel membranlar, bir buz banyosunda serbest radikal polimerizasyonu ile yarı dondurulmuş ortamda sentezlendi ve SEM/EDX, mikro-CT ve şişme oranı ölçümleri ile karakterize edilmiştir. In vitro biyoyumluluk, kültür edilmiş fibroblast hücreleri kullanılarak yapılan hücre yaşayabilirlik çalışmalarıyla değerlendirilmiştir.

Anahtar Kelimeler: 2-Metakriloksietil fosforilkolin; 2-Hidroksietil Metakrilat; Kriyojel.

1. Introduction

Cryogels with unique physical properties such as swelling ratio, pore size, pore interconnectivity, mechanical behavior can be tuned as polymeric scaffolds for promoting cell-biomaterial [1-3]. Cryogels which are a subclass of hydrogels are synthesized via cryogelation technique at subzero temperatures (typically between -5 and -20°C) yielding highly interconnected polymeric network [4]. Cryogels respond to external stimuli such as electrical [5], thermal [6], magnetic [7], and pH [8] etc. are promising materials for drug delivery and tissue engineering. Cryogels (can also have ionic, nonionic, amphoteric, or zwitterionic characters) are synthesized using synthetic and/or naturally derived polymers, and a polymer matrix incorporating materials such as nano, micro-, or macroparticles [9-14]. The extracellular matrix components can be also used to form biomimetic cryogels that exhibit improved biological and cell-adhesive features for tissue engineering applications.

Biomimicry of structural and biorecognition features of cellular components/sub-components provides to design novel synthetic biomaterials [15-17] which can be used in various biomedical applications such as diverse as tissue engineering [18], drug delivery [19], therapeutics [20], diagnostics [21], etc. Grafting or incorporation of zwitterionic

phosphorylcholine (PC) group containing moieties, a polar phospholipid presents in cell membranes, is one of the promising options to realize the biomimicry strategy [22-25]. A synthetic biomimetic molecule, 2-methacryloyloxyethyl phosphorylcholine (MPC) based polymeric materials have been widely used for tissue engineering and drug delivery systems to improve blood compatibility, resist protein adsorption, denaturation and cell adhesion, and prevent bacterial adherence [26]. The electrically neutral feature and formation of hydration shell surrounding the PC group provide these characteristics to MPC molecules [27-29]. Due to the reactivity of the methacrylate group, MPC can be easily copolymerized via various methods to develop numerous materials, having various applications in biomedical fields [22].

Herein, the present study on the influence of MPC on the in-vitro biocompatibility of poly(2-hydroxyethyl methacrylate) P(HEMA) cryogel membranes. P(HEMA) membranes cryogel membranes containing 0, 10, 20, and 30 mg of MPC were synthesized in a semi-frozen medium by free radical polymerization in an ice bath and characterized by SEM/EDX, micro-CT, and swelling ratio measurements. In vitro biocompatibility was assessed from cell viability studies performed using cultured fibroblast cells. So, it is aimed to develop bio-inspired, efficient and environmentally friendly MPC containing cryogel membranes which can be applied as polymeric scaffolds.

2. Materials and Methods

2.1. Materials

2-Methacryloyloxyethyl phosphorylcholine (MPC), N, N'-methylene-bis(acrylamide) (MBAAm), 2-hydroxyethyl methacrylate (HEMA), and ammonium persulfate (APS) were supplied by Sigma (St Louis, MO, USA). N, N, N', N'-Tetramethylene diamine (TEMED) was supplied from Fluka A.G. (Buchs, Switzerland). L929 fibroblast cell line was obtained from ŞAP Institute (Ankara, Turkey). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and trypsin-EDTA were purchased from Biological Industries (Cromwell-US). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Serva (Heidelberg, Germany).

2.2. Synthesis of p(HEMA) cryogel membranes

Briefly, HEMA (2.475 ml) and MPC (10, 20, and 30 mg separately) were dissolved in deionized water (2.525 mL). MBAAm (0.54 g) was dissolved in deionized water (DI) (10 mL). The two aqueous solutions were mixed and degassed. Total concentration of monomers was 20% (w/v). PHEMA cryogel membranes were synthesized between two glass plates at -16°C for 24 h

via free radical polymerization initiated by TEMED (30.0 µL) and APS (30.0 mg). After adding APS, the solution was cooled for 2 min. TEMED was added and the reaction mixture was stirred for 30 s. Right after, the four polymerization solutions containing 0, 10, 20, and 30 mg separately were poured between two glass plates (four different glass couples) separated with 2.0 mm thick spacer and were kept at -16°C for 24 h and following thawed at 25°C. After extensively washing with an excess of DI and pure ethanol until obtaining a clear washing solution, the cryogels were cut into circular membranes (0.5 cm in diameter). The cryogel membranes were stored in buffer at 4 °C.

2.3. Characterization of PHEMA cryogel membranes

Cryogel membranes were dried to constant weight (W_{dry}) in the oven at 50°C and soaked in DI in an isothermal water bath (25 ± 0.5 °C) for 2h. The swollen cryogel membrane was taken out from the aqueous medium and weighed ($W_{swollen}$, g) after carefully removing of water adsorbed on the surface. The swelling degree was calculated as:

$$SD\% = (W_{swollen} - W_{dry}) / W_{dry}$$

The morphology and chemical characterization of a cross-section of the cryogel membranes was analyzed by scanning electron microscope with energy-dispersive X-ray spectroscopy (SEM–EDX) (Gaia 3, Tescan, Czech Republic).

The internal structure of cryogel membranes in a non-destructive manner was investigated by micro-computed tomography (micro-CT) (Bruker, Skyscanner 1272). The cryogel membranes were scanned with a voxel size of 8.0 µm at a voltage of 30 kV and a current of 45 µA.

2.3. MTT cell proliferation assay

The reduction reaction of tetrazolium salts is a reliable option to evaluate cell proliferation. The tetrazolium MTT is reduced by metabolically active cells (by mitochondrial enzymes related to metabolic activity) to form reducing agents such as nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH). The resulting intracellular purple formazan can be quantified at 570 nm spectrophotometrically. The MTT cell proliferation assay measures the cell proliferation rate and inversely when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The tetrazolium ring in the solutions is broken down by dehydrogenase enzymes in mitochondria and forms purple colored formazan crystals. The color change observed in living cells gives the absorbance values using Elisa Plate Reader.

L929 fibroblast cells were cultured in 25 cm² culture flasks containing Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS), at 37°C with 5% CO₂. At 95% confluence, the cells were trypsinized and subsequently, resuspended in complete medium. L929 cells (10x10⁴ cell/mL) were seeded in 96-well plates and incubated overnight.

Cryogel membrane extracts were prepared for the determination of cryogel membranes cytotoxicity. According to the ISO 10993-5 standard, the concentration of 0.2 g/mL cryogel membranes were incubated into the cell culture medium at 37°C under 5% CO₂ stream for 72h. After the incubation, membrane extracts were pipetted onto the cell. Plates were incubated for 24 h at 37°C under 5% CO₂ stream. Fresh medium containing MTT (5.0 mg/mL) was pipetted to each well. Later 2 h of incubation at 37°C, 100 µL of MTT solvent (isopropanol-HCl) was added to the wells and absorbance was read at 570 nm in an ELISA plate reader. The measurements were studied in 8 replicates. Only the medium was used as the control group. The percent cell viability was calculated as below formula based on control groups.

$$\text{Viab. \%} = 100 \times \text{OD}_{570e} / \text{OD}_{570b}$$

OD_{570e}: Optical density of samples

OD_{570b}: Optical density of negative control groups

3. Results and Discussion

3.1. Characterization of PHEMA cryogel membranes

Scanning electron microscopy images of the highly and interconnected porous structure of the PHEMA and P(HEMA-MPC) cryogel membranes with nonporous bead like walls were given in Fig. 1. The pore size of the cryogel membranes depends on the amount of MPC incorporated to polymeric structure. The degree of porosity became smaller with increasing MPC amounts. MPC was incorporated at a varying amount of 10, 20, and 30 mg. SEM-EDX results also showed that the percentage of MPC in polymeric structures is proportional to these ratios if the atomic percentage of the phosphorus atom due to the existence of MPC in the polymeric structure was discussed Fig. 1.

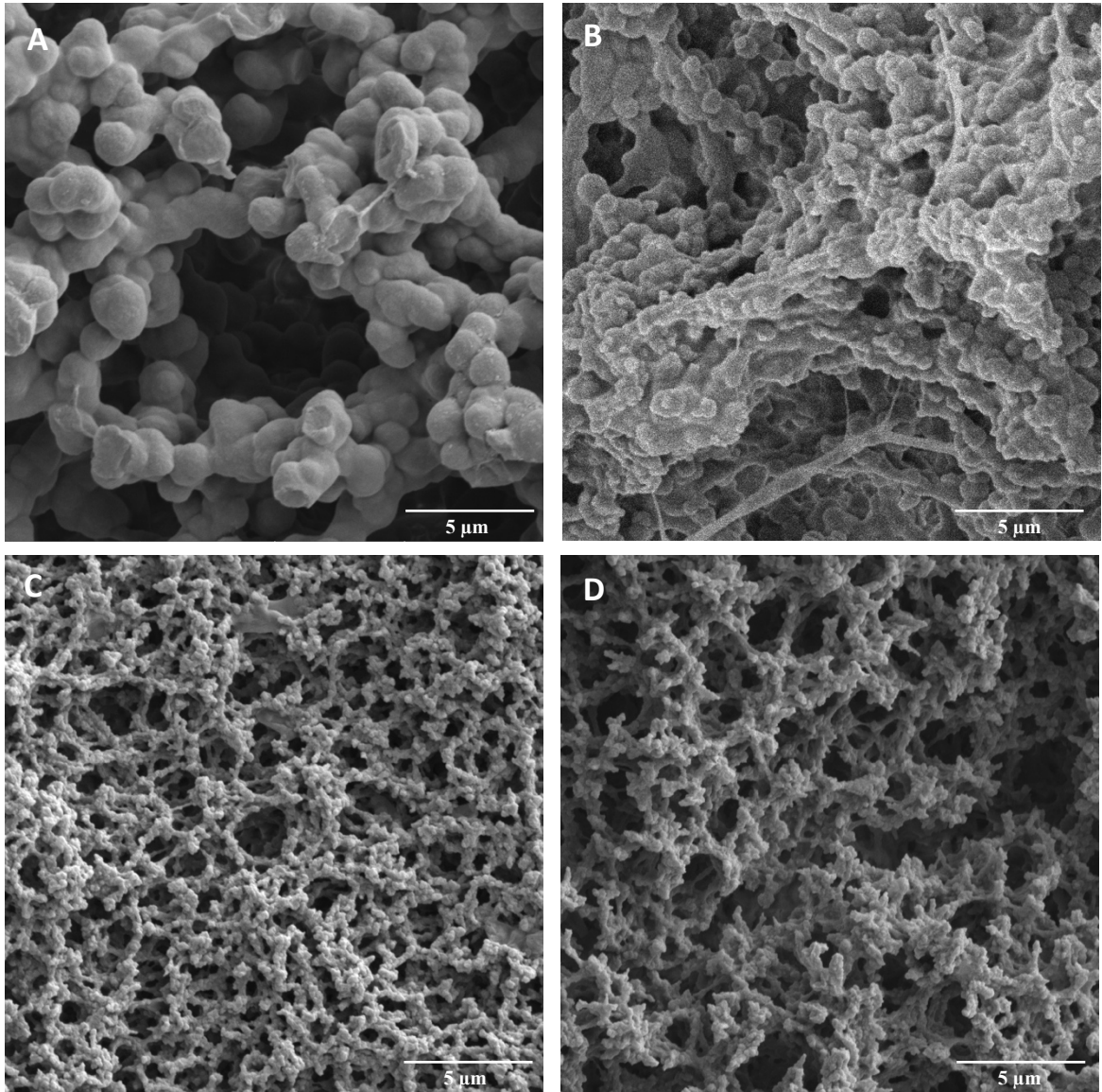


Figure 1: SEM images of PHEMA cryogel membranes containing MPC A) 0 mg, B) 10 mg, C) 20 mg, and D) 30 mg

The pore size ranged between 8.001-424.0034 μm , 8.001-296.0024 μm , 8.001-168.0015 μm , and 9.3001-46.5004 μm for PHEMA, PHEMA incorporated 10 mg of MPC, PHEMA incorporated 20 mg of MPC, and PHEMA incorporated 30 mg of MPC, respectively (at the voxel resolution of 8.0 μm). The range of pore size decreased with an increasing amount of MPC which indicated that the porosity feature of cryogel membranes shifted from super-macroporous to macroporous structure (Fig. 2).

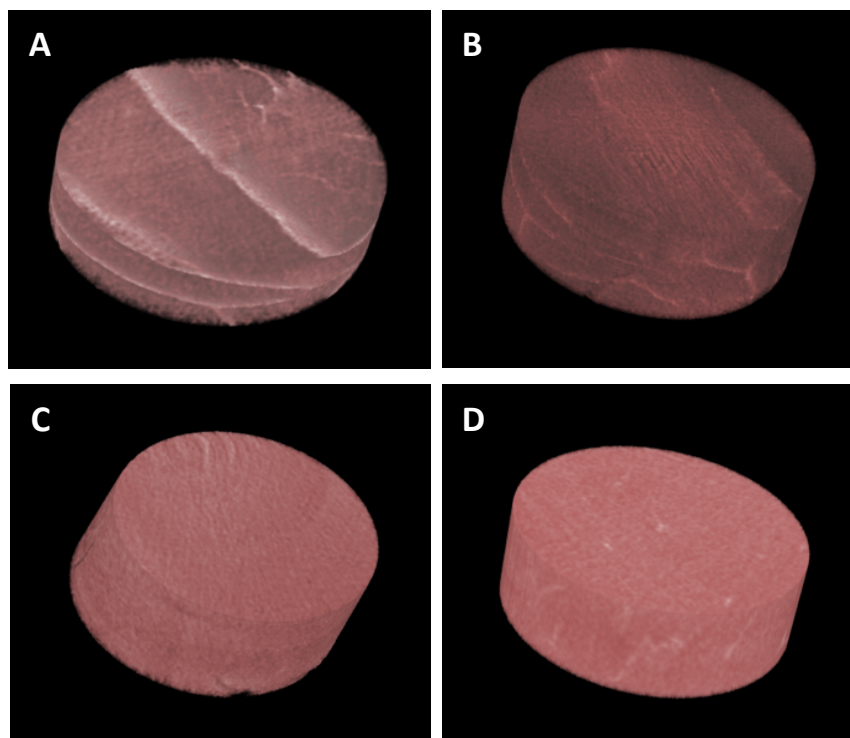


Figure 2: Micro-CT analysis reveals 3D reconstruction of PHEMA cryogel membranes containing MPC A) 0 mg, B) 10 mg, C) 20 mg, and D) 30 mg

The equilibrium swelling degrees of the bare PHEMA cryogel membrane and crosslinked P(HEMA-MPC) cryogel membrane containing 30 mg of MPC were 4.76 g H₂O/g cryogel and 6.71 g H₂O/g cryogel. The swelling degree of crosslinked P(HEMA-MPC) cryogel membrane was higher than the bare PHEMA cryogel membrane due to the high hydrating ability (derived from the electrostatic interactions as well as hydrogen bonds) of poly(MPC) chain inside the polymeric structure. Besides, a smaller porous structure reduced interconnected flow- channel while increasing specific polymeric surface. All types of cryogel membranes are opaque, sponge like squeezable and highly elastic. Water accumulated inside the pores of the cryogel membrane can be easily removed through compressing by hand.

The cryogel membranes with cells were washed with PBS buffer and fixed with 2.5% glutaraldehyde in PBS buffer for 1 h and immersed to sequential dehydration in graded ethanol (35, 50, 70, 80, 90, 100%) and were dehydrated with hexamethyldisilazane (HDMS). After drying, the samples were coated with gold/palladium mixture for SEM examination.

To the investigation of the cell adhesion on the cryogel membranes, L929 cells were cultured on the cryogel membranes as mentioned before. After 5 days of incubation periods, SEM images of cryogel membranes were taken (Fig. 3). According to the images, L929 cells adhered to the surface of the cryogel membranes containing 10, 20, and 30 mg of MPC (Fig. 3B, C, and

D). There was not seen a cell on the cryogel membrane A few cells were seen on the cryogel membrane B. A large number of cells adhered to the surface of the cryogel membranes containing 20.0 mg and 30.0 mg of MPC (Fig. 3C and D). Cellular extensions of outgrown cells were clearly seen as shown in Fig. 3C and D. The results indicate that increasing the MPC amount in the polymeric structure increased L929 cells outgrow on the cryogel membranes.

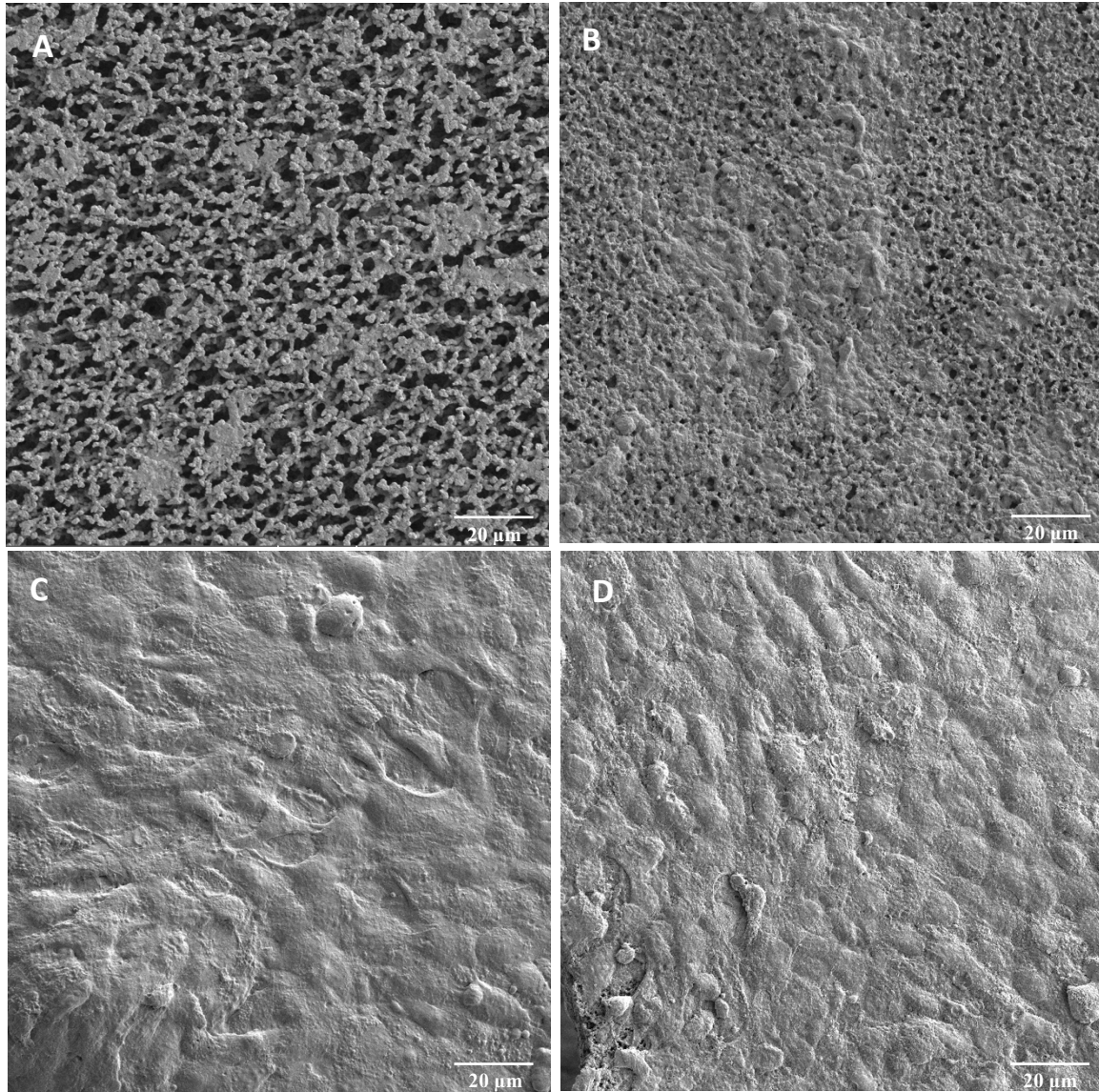


Figure 3: SEM images of L929 cells cultured on the PHEMA cryogel membranes containing MPC A) 0 mg, B) 10 mg, C) 20 mg, and D) 30 mg

The viability percentage of the L929 cells cultured on PHEMA cryogel membrane containing 0 mg of MPC was found $51.5\% \pm 2.81\%$. The viability percentages of the L929 cell line cultured on PHEMA cryogel membranes containing 10, 20, and 30 mg of MPC were found $53.83\% \pm 1.43\%$, $72.92\% \pm 1.94\%$, and $93.85\% \pm 2.22\%$, respectively. There was a statistically

significant difference in cell viability of PHEMA cryogel membrane containing 10 mg and 20 mg MPC compared with the negative control group ($P < 0.05$). There was no significant difference in cell viability between PHEMA cryogel membrane containing 30 mg MPC with the negative control group ($P > 0.05$). As a result of the cell viability studies, the copolymerization of MPC residue with HEMA increases the biocompatibility of the PHEMA cryogel membranes ($P < 0.05$). When PHEMA cryogel membranes containing MPC were compared among themselves, the viability percentage of the L929 cell line increased with the increasing amount of MPC ($P < 0.05$) (Fig. 4).

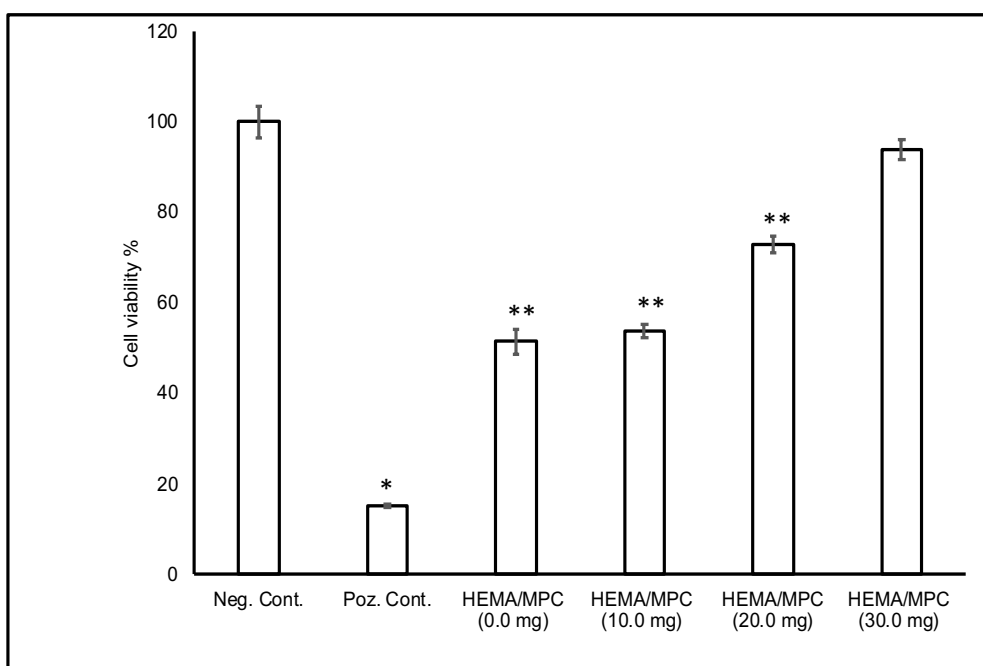


Figure 4: Indirect cytotoxicity of PHEMA membranes with different MPC amount with L929 cells (** $P < 0.05$)

4. Conclusion

Phospholipid based biomimicry has been applied to obtain improved in vitro biocompatibility of P(HEMA) cryogel membranes through the copolymerization of MPC and HEMA in a semi-frozen medium by free radical polymerization. P(HEMA) cryogel membranes containing 20.0 mg and 30.0 mg of MPC exhibit the significant characteristics for increased L929 cells outgrow ($P < 0.05$). A large number of cells adhered to the surface of the cryogel membranes containing 20.0 mg and 30.0 mg of MPC. The viability percentages of the L929 cell line cultured on PHEMA cryogel membranes were found as $72.92\% \pm 1.94\%$ and $93.85\% \pm 2.22\%$, respectively. According to the SEM/EDX, micro-CT, swelling ratio measurements, and cell viability studies, it was developed bio-inspired, efficient, and environmentally friendly cryogel membranes which can be applied as polymeric scaffolds.

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