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Macroporous hydrogels based on 2-hydroxyethyl methacrylate. Part 4: Growth of rat bone marrow stromal cells in three-dimensional hydrogels with positive and negative surface charges and in polyelectrolyte complexes

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Abstract The growth of bone marrow stromal cells was assessed *in vitro* in macroporous hydrogels based on 2-hydroxyethyl methacrylate (HEMA) copolymers with different electric charges. Copolymers of HEMA with sodium methacrylate (MA^-) carried a negative electric charge, copolymers of HEMA with [2-(methacryloyloxy)ethyl] trimethylammonium chloride ($MOETA^-$) carried a positive electric charge and terpolymers of HEMA, MA^- and $MOETA^+$ carried both, positive and negative electric charges. The charges in the polyelectrolyte complexes were shielded by counter-ions. The hydrogels had similar porosities, based on a comparison of their diffusion parameters for small cations as measured by the real-time tetramethylammonium iontophoretic method of diffusion analysis. The cell growth was studied in the peripheral and central regions of the hydrogels at 2 hours and 2, 7, 14 and 28 days after cell seeding. Image analysis revealed the highest cellular density in the HEMA-$MOETA^+$ copolymers; most of the cells were present in the peripheral region of the hydrogels. A lower density of cells but no difference between the peripheral and central regions was observed in the HEMA-MA^- copolymers and in polyelectrolyte complexes. This study showed that positively charged functional groups promote the adhesion of cells.

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Introduction

Macroporous hydrogels with communicating pores are a class of materials formed by a three-dimensional polymer network with pore sizes between 10–100 μm. They have a large surface available for cellular ingrowth and are strongly hydrated in a physiological solution. They are in many ways similar to the environment in developing nervous tissue [1]; they are capable of providing mechanical support to ingrowing cells and axons, and their chemical (e.g. composition and charge) and physical properties (e.g. porosity, pore size, surface and mechanical properties) can be tailored to a specific use. The usefulness of macroporous hydrogels based on N-(2-hydroxypropyl)-methacrylamide (HPMA) and 2-hydroxyethyl methacrylate (HEMA) as implants into the brain or spinal cord has been investigated for more than ten years [1–4].

Bone marrow stromal cells (MSCs) are pluripotent progenitor cells that have the capability to migrate and exhibit site-dependent differentiation in response to environmental signals. In culture they can differentiate into osteoblasts, chondrocytes, adipocytes and myoblasts [5, 6]. After transplantation into the brain, MSCs respond to intrinsic signals and differentiate *in vivo* into various types of glial cells, in some experiments, also into neurons and may rescue partly damaged cells and accelerate regeneration by production of growth factors [7–9]. The use of MSCs in cell therapies may have some advantages over the use of other sources of cells: they are relatively easy to isolate, they may be used

in autologous transplantation protocols and bone marrow as a source of cells has been already approved for the treatment of hematopoietic diseases. It was shown that transplanted human MSCs have the capacity to increase the expression of growth and trophic factors in the ischemic rat brain [10]. Recently, several attempts were made to create cell-polymer constructs with genetically engineered cells [11] or with stem cells [12, 13] to provide a biohybrid system for tissue bridging in cases where a large tissue defect exists.

The influence of surface charge on cell growth has been studied since 1975 in order to provide better cultivation conditions in culture flasks. It is now well understood that many cell types, including neuroblastoma cells [14], adhere better to positively charged surfaces. Enhanced MSC attachment and suppressed spreading were observed on a positively charged indium tin oxide (ITO) pattern-coated substrate [15]. However, in all previous studies, only two-dimensional growth patterns were examined. In our study, we have focused on evaluating three-dimensional cell growth in macroporous hydrogels; this is an attractive material for implantation into the CNS after an injury.

Materials and methods

Four series of macroporous hydrogels based on crosslinked copolymers of 2-hydroxyethylmethacrylate (HEMA) were prepared. The crosslinked copolymer of HEMA with methacrylic acid in the sodium salt form (MA^-) (Series 1) was prepared according to [16]. The crosslinked copolymer of HEMA with [2-(methacryloyloxy)ethyl]trimethylammonium chloride ($MOETA^+$) (Series 2), the terpolymer of HEMA with MA^- and $MOETA^+$ (Series 3) and the polyelectrolyte complex of HEMA-MA^- crosslinked copolymer with linear poly($MOETA^+$) (Series 4) were prepared similarly in a pelleting apparatus in the presence of fractionated sodium chloride particles [16, 17]. The average pore size in all the prepared hydrogels was 40 μm. The hydrogels after polymerization were thoroughly washed with water and saline (2 weeks) in order to remove unreacted monomers and solvent, autoclaved (120°C, 20 minutes) and cut into blocks approximately 2 × 2 × 2 mm (in sterile conditions). These blocks were placed into Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin (MSC culture medium) overnight.

For the isolation of rat MSCs, femurs were dissected from 4-week-old Wistar rats. The ends of the bones were cut, and the marrow was extruded with 5 ml of DMEM using a needle and syringe. Marrow cells were plated in 80 cm^2 tissue culture flask in MSC culture medium. After 24 hours, the non-adherent cells were removed by replacing the medium. The medium was replaced every 2–3 days as the cells grew to confluence. The cells were lifted by incubation with 0.25% trypsin (GIBCO) and passaged at a density of 6000/cm^2. After 4–7 passages, the cells were stained with the membrane fluorescent dye PKH26 (PKH26-GL, Sigma Aldrich, Germany) and diluted to obtain a concentration of 10^5 cells per milliliter. The blocks of hydrogel were placed into Petri-dishes containing the cell suspension and left for one hour on a rocker. After this procedure, the hydrogels were washed with phosphate buffered saline (PBS) and placed into a Petri-dish with MSC culture medium.

Cell density in the hydrogels was evaluated 2 hours after incubation and on the 2^{nd}, 7^{th}, 14^{th} and 28^{th} days of cultivation. Each block of hydrogel was removed from the medium, washed with PBS and mounted in 3% agarose gel. Then, three 400-μm thick slices were cut from the middle of each hydrogel block, and the slice images were taken using an AxioScope fluorescent microscope (Carl Zeiss, Germany). The approximate cell count per mm^2 of the slice (cellular density) was calculated in the peripheral (up to 100 μm from the border of the slice) and central (more than 150 μm from the border of the slice) areas of the hydrogel using the image analysis toolbox in Matlab 6.1 (The MathWorks, Inc.) by dividing the total fluorescence intensity of the area by the average fluorescence intensity of a single cell and the measured area size. This procedure was used in order to correct for the relative decrease in the concentration of the membrane dye PKH26 during cell growth and division.

To measure the diffusion parameters in the hydrogels, we used the real-time iontophoretic tetramethylammonium (TMA^+) method [18]. The pore volume fraction α (α = pore volume/total hydrogel volume) and tortuosity $\lambda = (D/ADC)^{0.5}$ (where D is the free TMA^+ diffusion coefficient and ADC is its apparent diffusion coefficient in hydrogel) were measured in all the hydrogels.

All data are expressed as the mean ± the standard deviation. The quantitative results from the cell counts were analyzed by the student's t-test. The results of statistical analysis were accepted as significant if p-values were less than 0.05.

Results and discussion

The main difference in the properties of the hydrogels employed in this study was the presence of differently charged polar groups on their surfaces. The copolymers of Series 1 had negative surface charges, caused by the presence of carboxylic groups; the copolymers of Series 2 had positive charges, caused by the presence of quarternary ammonium groups. The terpolymers of Series 3 and the polyelectrolyte complexes of Series 4 had both positive and negative charges

Table 1 The diffusion parameters (volume fraction α and tortuosity λ) of the four series of hydrogels before cell seeding. The high values of the volume fraction α represent the large space available for cell growth within the hydrogels; low values of the tortuosity λ indicate a very small number of diffusion barriers. The p-values showed no significant difference ($p > 0.05$) in the diffusion properties between the four series of hydrogels.

	α	λ	n
Series 1 (HEMA-MA$^-$, negative charge)	0.84 ± 0.02	1.06 ± 0.01	10
Series 2 (HEMA-MOETA$^+$, positive charge)	0.87 ± 0.07	1.03 ± 0.04	5
Series 3 (HEMA-MA$^-$-MOETA$^+$, both charges)	0.86 ± 0.04	1.08 ± 0.02	10
Series 4 (polyelectrolyte complexes)	0.82 ± 0.03	1.14 ± 0.01	11

in their polymer chains. The difference between Series 3 and 4 was that the counter-ions of the Series 3 hydrogels were low-molecular weight (Na^+, Cl^-), while the Series 4 hydrogels had counter-ions bound on a macromolecular chain of linear poly(MOETA$^+$). Therefore, the Series 4 hydrogels' charges were strongly shielded by the polymer counter-ion, resulting in properties similar to those of uncharged hydrogels, as reported previously [17, 19].

We measured the diffusion properties of the hydrogels by the real-time iontophoretic (TMA$^+$) method [18, 20], which was utilized in previous studies to measure the properties of neural tissue [21, 22] and HPMA based hydrogels [1]. The diffusion measurements did not show significant differences ($p > 0.05$) in the pore volume or tortuosity between any of the hydrogels of Series 1–4 (Table 1). The diffusion properties of the hydrogels indicate that a large space is available for cell ingrowth and that the diffusion of small cations is minimally hindered within the hydrogels.

Each hydrogel block was placed in a 0.5 ml suspension of MSCs in medium (10^5 cells per 1 ml) for one hour, then the flasks with the hydrogels floating within the cell suspension were placed on a rocker for one hour. Since the hydrogel blocks were floating within the cell suspension, the cells were able to enter the hydrogels from all sides of the hydrogel blocks by passive cell displacement; as a result, the concentration of cells in the peripheral areas after an hour of rocking was comparable to the concentration of the cells in the medium. The large average porosity of the hydrogels that were used (40 μm) and their communicating pores allowed the cell suspension to flow throughout the hydrogels, therefore some cells were immediately found in the center of the gels. However, their number was low. During the subsequent incubation period (which lasted one hour to 28 days), the cells moved within the hydrogel blocks predominantly by active migration. The combination of these two types of cellular movement through the pores created the resultant distribution of MSCs within the hydrogels. Some MSCs were present in the central regions of all the examined hydrogels as early as 2 hours after incubation, and the number of cells in the central regions increased over time. This demonstrates that all of the hydrogels contain communicating pores and that MSCs are able to move through these pores *in vitro*.

The cellular density (expressed as approximate cell count per mm^2 of the slice) was lowest on the first day of incubation. The density of cells then increased on the 2nd and 7th days, indicating the cellular growth within the hydrogels. The highest cellular density was observed on the 7th day of cultivation; the cellular density then decreased and reached an approximately steady state on the 14th and 28th days of cultivation (Fig. 1A).

The increase in cellular density was caused by cell growth within the hydrogel. The decrease that occurred after the 7th day of cellular growth within the hydrogel was probably due to cell apoptosis. The reason for the apoptosis could be either the reduced availability of nutrients or glucose in the hydrogels or the increased contact between cells after reaching a critical cellular density. This issue will be further studied by using a perfusion chamber or bioreactor for cell cultivation in the hydrogels.

Starting the 2nd day after cell seeding, the MSCs were distributed in two ways in the hydrogels (Fig. 2). In Series 1 (HEMA-MA$^-$ copolymer) and in Series 4 (polyelectrolyte complexes), there was no significant difference between the cellular density in the central region and the density in the peripheral areas ($p > 0.05$); the cells were spread uniformly throughout the hydrogels. However, in Series 2 and Series 3 (HEMA-MA$^-$-MOETA$^+$ terpolymer and HEMA-MOETA$^+$ copolymer), there was a significantly greater cellular density ($p < 0.05$) in the peripheral regions than in the central region of the hydrogel block (Fig. 1B–E).

The different behaviour of MSCs in the Series 2 and 3 hydrogels is probably mediated by the adsorption of serum macromolecules onto the positively charged quarternary ammonium groups. This adsorption increases the attachment of MSCs to the surface of the hydrogel, leading to enhanced cell attachment and suppressed cell migration, similar to Qiu's observations on differently charged ITO surfaces [15]. This behaviour results in a higher cellular density in the peripheral regions of the hydrogel. It can be noted that the polyelectrolyte complexes of the Series 4 hydrogels – although they contain both positive and negative

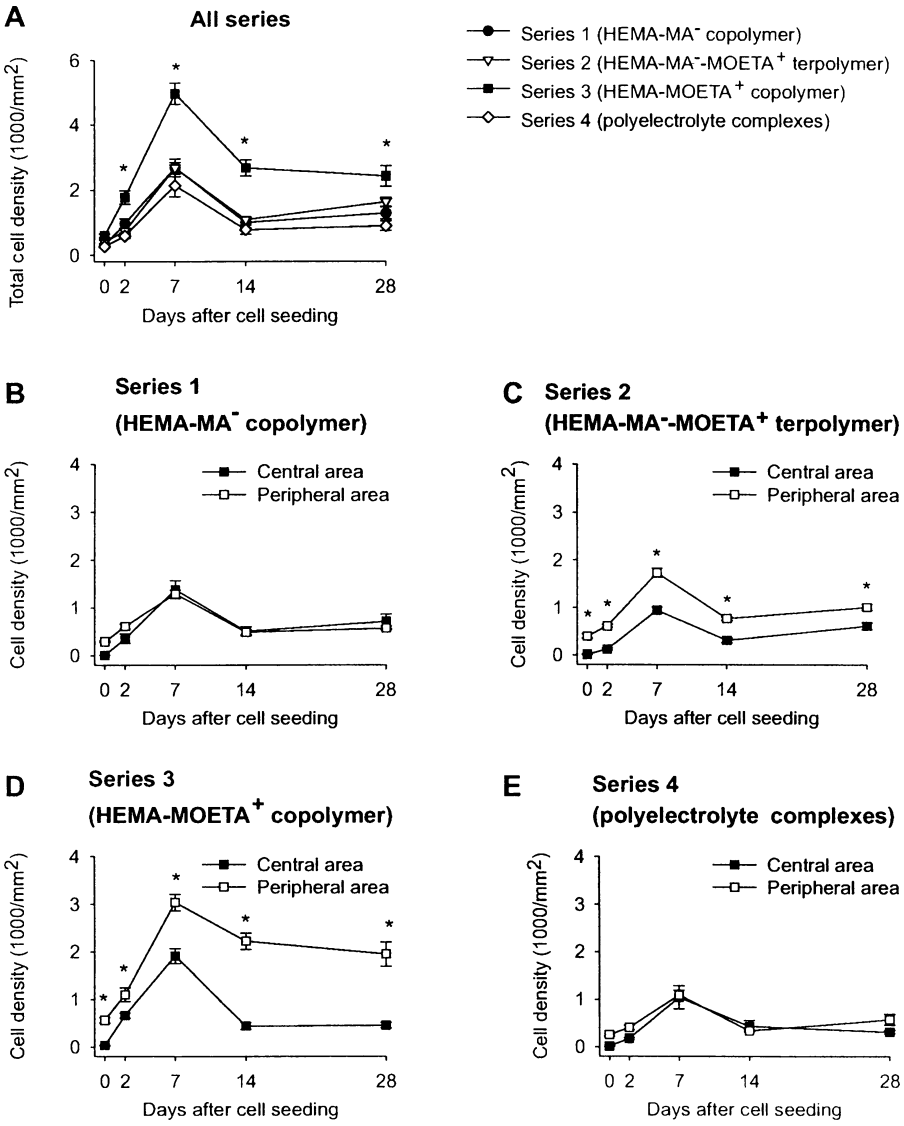

Fig. 1 A: The cellular density, approximated as the number of cells per mm^2, on the day of implantation and the 2nd, 7th, 14th and 28th day after cell seeding in the four groups of tested hydrogels. The highest cell count ($p < 0.05$) was found in the Series 3 hydrogel (HEMA-MOETA$^+$ copolymer) beginning on the 2nd day after cell seeding (marked by asterisks). B, C, D, E: The cellular density, approximated as thousands of cells per mm^2, in the central (dark squares) and peripheral (white squares) regions of the four groups of hydrogels on the day of implantation and the 2nd, 7th, 14th and 28th day after cell seeding. Significant differences ($p < 0.05$) between the central and peripheral parts, which were found in Series 2 (HEMA-MA$^-$-MOETA$^+$ terpolymer) and Series 3 (HEMA-MOETA$^+$ copolymers), are marked by asterisks.

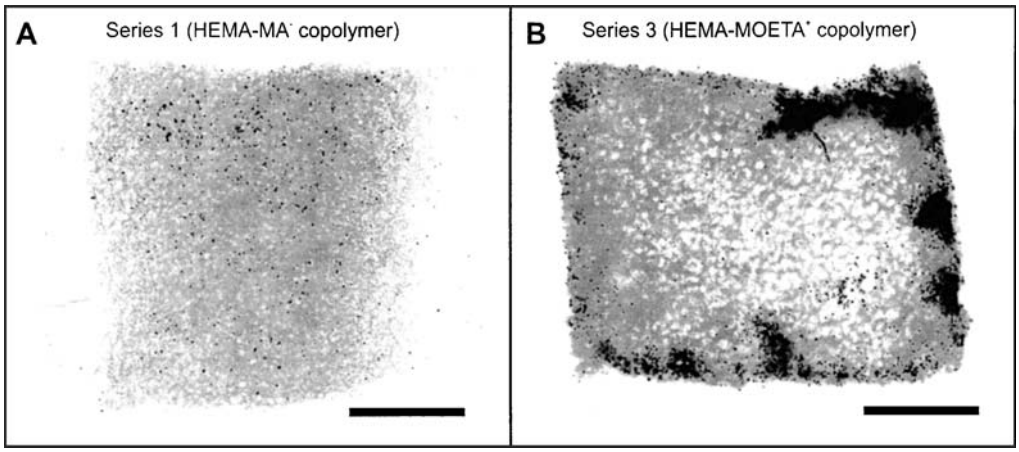

Fig. 2 The two observed distributions of MSCs within the hydrogels on the 28th day after implantation: the cells are represented by dark dots (bar = 500 μm).
A: The cells are uniformly scattered throughout the Series 1 hydrogel (HEMA-MA$^-$ copolymer). The same cell distribution was also observed in the Series 4 (polyelectrolyte complexes).
B: The cells are concentrated in the peripheral region of the Series 3 hydrogel (HEMA-MOETA$^+$ copolymer). The same cell distribution was also seen in the Series 2 (HEMA-MA$^-$-MOETA$^+$ terpolymer).

charges — behave from a biological point of view similarly to the Series 1 copolymers, which contain only negative charges and do not adsorb serum macromolecules. This finding might be caused by the lower availability of positively charged functional groups in the polyelectrolyte complexes than in the HEMA-MOETA$^+$ copolymer and HEMA-MA$^-$-MOETA$^-$ terpolymer; positive charges in the polyelectrolyte complex are strongly shielded by macromolecular counter-ions (see above).

Our recent findings have shown that transplanted MSCs have the capacity to improve functional outcome in rats with a spinal cord injury [23]. The tested hydrogels can also serve as material for bridging CNS defects [2]. In addition, they can be used as cell carriers for *in vivo* experiments examining gel implantation into spinal cord lesions [24], and the use of these gels can be tailored according to particular conditions. The good adhesion and rapid proliferation observed with positively charged HEMA-MOETAC1 and HEMA-MA-MOETAC1 gels are suitable for the long term *in vitro* culturing of cell-carrier constructs prior to implantation, while the more even distribution combined with lower cell adhesion (HEMA-MA gels) is suitable for stem cell delivery into a lesion site. Polymer hydrogels can be a useful tool in tissue engineering strategies.

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