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# Surface modification of hydrogels based on poly(2-hydroxyethyl methacrylate) with extracellular matrix proteins

**Eduard Brynda · Milan Houska · Jiří Kysilka · Martin Přádný · Petr Lesný · Pavla Jendelová · Jiří Michálek · Eva Syková**

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**Abstract** Infrared attenuated total reflection spectroscopy was used for in situ observation of the deposition of collagen I on poly(2-hydroxyethyl methacrylate-co-methacrylic acid, 2.9%) hydrogels and subsequent attachment of laminin or fibronectin on the collagen surface. While there was no adsorption of collagen dissolved in an acid solution on the hydrogel surface, it deposited on the surface at pH 6.5. The collagen layers with attached laminin or fibronectin were stable on hydrogel surface in physiological solution. The modification with collagen and particularly with collagen and laminin or fibronectin allowed the adhesion and growth of mesenchymal stromal cells and astrocytes on the hydrogel surface.

## 1 Introduction

Tissue engineering uses polymer scaffolds seeded with cells to repair damaged tissues and organs. The scaffold should support cell adhesion and function leading to the formation of new tissue. Synthetic hydrogels are convenient materials for many applications owing to their mechanical properties similar to extracellular matrix (ECM) and soft tissues [1, 2]. The hydrogel structure containing a considerable amount of water allows easy transport of gasses, ions, and low-molecular-weight nutrients and waste products. Hydrogels can be prepared in various shapes and porosity, and with mechanical properties controlled by crosslinking. Their softness facilitates low invasive implantation. On the other hand, the highly hydrophilic hydrogel surface does not readily promote cellular adhesion and prevents considerably adsorption of specific cell-interactive ECM proteins such as laminin, fibronectin, and vitronectin [3].

From the tissue engineering point of view, PHEMA-based hydrogels could be suitable for fabrication of permanent non-degradable or degradable and shape-stabilizing scaffolds. Relatively high water content (37–96 wt%) makes the mechanical properties of homogeneous cross-linked PHEMA-based hydrogel similar to those of living soft tissues. Thus, PHEMA hydrogels do not irritate mechanically and frictionally the surrounding tissue [4, 5]. Also permeability of the gels to low-molecular-weight metabolites is similar to that of living tissue. They can be prepared with various degrees of porosity, with pore sizes from hundreds of nanometers to hundred micrometers in sponges with communicating intermediate micropores [4, 6–9], and sterilized with heat and steam. By contrast, poor cell adhesion and low protein adsorption on the hydrogel surface disable the scaffolds for the efficient cell seeding [6, 10]. Copolymerization of HEMA with hydrophobic methyl methacrylate or ethyl methacrylate improved significantly the cell adhesion and spreading onto the hydrogel surface [6]. The results indicated a relation between this improvement and adsorption of fibronectin to the copolymer surface [10, 11]. Generally, the decreased

E. Brynda (✉) · M. Houska · J. Kysilka · M. Přádný · J. Michálek
Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, 162 06 Prague 6, Czech Republic
e-mail: brynda@imc.cas.cz

E. Brynda · M. Přádný · P. Lesný · P. Jendelová · J. Michálek · E. Syková
Center for Cell Therapy and Tissue Repair, Charles University, 150 06 Prague 5, Czech Republic

P. Lesný · P. Jendelová · E. Syková
Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, 142 20 Prague 4, Czech Republic

hydrophilicity of the copolymer is associated with a decrease in hydrogel/water interfacial energy promoting cell adhesion and adsorption of fibronectin and other proteins. This approach may not be optimum for hydrogel scaffolds, because a decrease in bulk water content impairs desirable mechanical properties and permeability of the gels. In other in vitro experiments, the cell attachment increased on positively charged surfaces of hydrogels prepared by copolymerization of HEMA with [2-(methacryloyloxy) ethyl]trimethylammonium chloride [10, 12] or by covalent grafting the adhesion peptide sequence GRGDS on the surface of copolymers HEMA with methacrylic acid [12] or by positioning fibronectin between PHEMA chains [13].

In this work, a technique for coating of surfaces of PHEMA-based hydrogel scaffolds with a collagen layer, and subsequently modification by attachment of fibronectin or laminin is described. The surface modification improved considerably adhesion and growth of cells on the originally inert hydrogel surface. The procedure consists only in successive incubation of gel surfaces with various aqueous solutions. Thus hydrogel objects of any shapes and sizes including fibers or macroporous materials beads could be additionally modified.

## 2 Experimental

### 2.1 Chemicals

Collagen I, type A from rat tail, was from BD Bioscience (stock solution 4.66 mg/ml 0.02 M acetic acid), fibronectin (FN), from human plasma, was from Roche Applied Science, laminin (LM), from Engelbreth-Holm-Swarm murine sarcoma, basement membrane, and Darocur 1173, 1-(2-hydroxy phenyl)-2-methylpropan-1-one, were from Sigma. Methacrylic acid, 2-hydroxyethyl methacrylate, and ethylene glycol dimethacrylate were from Fluka. Polystyrene of $M_n$ 92,100 and MW distribution $M_w/M_n = 1.68$ was from Polymer Source, Inc.

Cell culture medium (DMEM) 1% penicilin/streptomycin (P/S) and fetal calf serum (FCS) were from Invitrogen, Inc. Poly-L-lysine, MW 300000, for coating the tissue plastic was from Sigma, phalloidin, Alexa Fluor 488 Conjugate was from Cambrex, Inc.

Phosphate-buffered physiological saline (PBS), pH 7.4, phosphate buffer 0.067 M (PB), pH 6.5 or 7.6 were used for deposition of proteins on substrates. Collagen I was deposited on substrate surfaces from solutions prepared by diluting stock solution with 0.005 M HCl or with PB, pH 6.5, to concentration of 50 μg collagen I per 1 ml. FN and LM were deposited from PBS.

### 2.2 Cell cultures

Mesenchymal stromal cells (MSC) were obtained by perfusion of the rat bone marrow and cultivated under standard conditions (37°C, 5% $CO_2$) in standard media (DMEM + 10% FCS + P/S). Isolation of cells was based on their adhesion to tissue cultivation plastic.

Astrocytes were obtained from brain tissue extract from newborn rat's marrow and cultivated under standard conditions (37°C, 5% $CO_2$) in standard media (DMEM + 10% FCS + P/S). Isolation of the cells was based on their adhesion to tissue cultivation plastic coated with poly(L-lysine).

### 2.3 Poly(HEMA/MANa) hydrogel plates

Poly(HEMA/MANa) hydrogel plates were prepared by crosslinking radical copolymerization of HEMA (9.66 g), methacrylic acid (MA) (0.35 g), ethylene glycol dimethacrylate (0.20 g) initiated by 2,2′-azobis(isobutyronitrile) (0.10 g) in poly(ethylene glycol) MW 400 (20 g). The polymerization mixture was filled between two polypropylene plates (10 × 10 $cm^2$, distance 0.1 cm) in a thermostatted aluminium form and polymerized at 80°C for 16 h. After polymerization the cross-linked PHEMA (3.4 wt% MA) plate was rinsed with 1% solution of sodium hydroxide (5 × 8 h), thus transforming the carboxyl groups of methacrylic acid to carboxylate groups, and then rinsed with physiological saline (5 × 8 h). Finally, the hydrogel was steam sterilized with steam at 120°C for 30 min.

### 2.4 Poly(HEMA/MA) formulation for infrared reflection spectroscopy

Poly(HEMA/MA) formulation for infrared reflection spectroscopy was prepared by red-ox initiated free radical polymerization of HEMA (2.76 g), MA (0.08 g) using ammonium persulfate (0.026 g) and sodium disulfite (0.026 g) in water-ethanolic solution (16 g ethanol, 4 g water) for 5 days at room temperature.

The surface of ZnSe reflection element was spin-coated at 800 rpm for 90 s with a solution consisting of the poly(HEMA/MA) formulation (0.5 ml), ethanol (2.5 ml), methylcellosolve (2.5 ml), UV initiator Darocur 1173 (2 μl), and crosslinking agent ethylene glycol dimethacrylate (2 μl). The spin-coated poly(HEMA/MA) (2.9 wt% MA) film on the reflection element was crosslinked by irradiating film with mercury UV lamp (500 W) for 5 min. Homogeneity of the coating and its roughness and uniformity were checked using optical microscopy and by visual observation of the drop pattern formed on the film surface

by condensation of water vapor when the film was exposed to water steam.

### 2.5 Hydrogel film thickness by atomic force microscopy

Dry poly(HEMA/MA) film spin-coated on ZnSe reflection element was scratched with a polystyrene spike. Thicknesses of about 40 nm for the dry film and about 90 nm for the film in PBS were evaluated by measuring the vertical distance between the film surface and bottom of the scratch using a multimode atomic force microscopy (AFM) Nanoscope IIIa (Digital Instruments).

### 2.6 Fourier-transform infrared attenuated total reflection spectroscopy

Attenuated total reflection (ATR) experiments were performed using Bruker IFS 55 spectrometer with a reflection attachment (Wilks Sci.) and a ZnSe reflection element (SPT, 45°, Harrick Sci. EM2121) spin-coated with a thin poly(HEMA/MA) film as described above. The experimental arrangement for the in situ measurements in aqueous solutions is shown in Fig. 1. The coated ZnSe reflection element was fixed in a flow cell in which the solutions were changed. An efficient monitoring of processes at the interface between aqueous solutions and the polymer film requires the film thickness substantially lower than the penetration depth of an evanescent electromagnetic wave of the totally reflected light penetrating from the reflection element into the adjacent medium of a lower refractive index [14]. The penetration depth of light at 1550 $cm^{-1}$ for ZnSe/45° and water was calculated as 0.97 μm. The integral intensity of the IR absorption band at 1725 $cm^{-1}$ (Fig. 2a) was used as a relative measure of the deposited poly(HEMA/MA) mass. The washing of water-soluble impurities and stability of the film were monitored after filling the flow cell with PBS. PBS in the cell was replaced four times in the first 4 h and again after incubation of the film overnight. Assuming that the film thickness was proportional to the mass, a correlation between the intensity of the band at 1725 $cm^{-1}$ and the film thickness measured by AFM was evaluated. Thickness of individual polymer films was then checked by the intensity of the band at 1725 $cm^{-1}$ and chosen empirically, so that the spectra of protein addlayer could be well detected. The attachment of proteins from solutions on poly(HEMA/MA) surface was monitored in situ by measuring amide II band at 1548 $cm^{-1}$ at suitable time intervals after introducing protein solution into the flow cell. Protein spectra (Fig. 2b) were obtained by subtracting the spectral background measured before the protein solution was introduced into the cell. To estimate an amount of collagen I deposited on the polymer surface, the surface was washed with the buffer and water. Then, the reflection element was taken out of the flow cell and dried in air. An integral intensity of the protein band at 1548 $cm^{-1}$ measured on the dried sample was compared with that measured on a sample on which 2 μg/$cm^2$ collagen was deposited by drying collagen solution. For the calibration, a layer (thickness 0.2 mm) of collagen solution 20 μg/ml (obtained by diluting the stock solution with water) was spread on the surface of a polymer film coated on the reflection element. Then the sample was stored in ammonia vapor for 5 min and dried in air. An error of 14% in such calibration was calculated from four experiments. A comparative experiment with collagen adsorption on polystyrene surface was performed using a ZnSe reflection

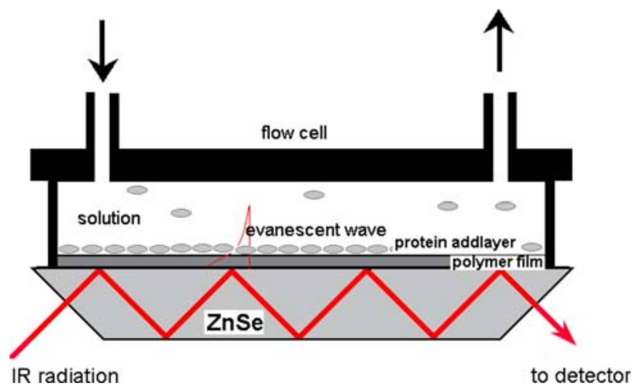


**Fig. 1** The schematic representation of the IR ATR measurement of protein deposition on polymer films

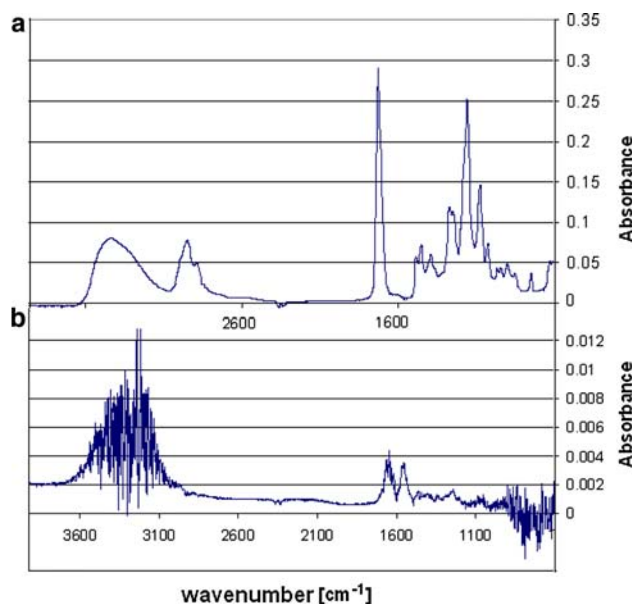


**Fig. 2** IR ATR spectrum of poly(HEMA/MA) film spin-coated on a ZnSe reflection element (**a**) and IR ATR spectrum of a collagen I layer on the poly(HEMA/MA) film in PBS obtained by subtracting background measured before collagen deposition (**b**)

element spin-coated with polystyrene film (thickness 90 nm).

### 2.7 Preparation of samples for cell-seeding

Poly(HEMA/MANa) hydrogel plate, prepared as described above, was incubated with phosphate buffer 0,067 M, pH 7.6 (PB) for 1 day and then the discs of a diameter 15 mm were cut off from the plate. The discs were incubated with solution of collagen I, 50 μg/ml 0,005 M HCl for 12 h. The amount of collagen solution was empirically selected so that the resulting equilibrium pH 5.5–6.5 of collagen was reached after about 1 h. The equilibrium pH resulted from the buffering of the initial acid collagen solution with PB contained in the swollen hydrogel discs. After 1 h, the discs were washed with PBS 10 times during 6 h. A part of the samples was further modified by incubation with laminin solution 50 μg/ml PBS or with fibronectin solution 50 μg/ml PBS for 12 h. Then the discs were washed with PBS and put in wells of tissue culture plates (Polystyrene Non-Tissue Treated Plate, 24 Well, Falcon Multiwell$^{TM}$). Four identical samples of one type were put into one of the four-wells column in a tissue culture plate. The whole procedure was performed in a laminar flow box at room temperature. Aqueous solutions for preparation of protein solution and washing the samples were filtered with 0.2 μm filters. The complete samples were sterilized by UV irradiation.

### 2.8 Cell-seeding

Phosphate-buffered physiological saline in the wells containing samples was gently sucked up and replaced with 950 μl of the cell culture medium to which 50 μl cell suspension of $2 \times 10^5$ cells/ml was added and gently stirred. The culture plates were then kept in an incubator at 37°C, 5% $CO_2$.

After 4 days, the cell suspension was sucked up and each surface was washed twice with PBS at room temperature. Subsequently, the cells were fixed in a 4% solution of paraformaldehyde for 20 min at 4°C, the surface was washed with PBS. The cells were stained for F-actin with the Phalloidin, Alexa Fluor 488 Conjugate, using the manufacturer's protocol. The cells were photographed using an inverted fluorescent microscope connected to the image analysis workstation (Carl-Zeiss). The average area of the adhering cell was computed using the Sigma Scan analysis software (SPSS).

## 3 Results and discussion

The development of an optimum technique for preparation of poly(HEMA/MA) films used in IR ATR was based on the evaluation of properties of films prepared in various ways. A maximum thickness of the film was limited by the applicability of ATR methods, i.e., it must be thin enough in comparison with the penetration depth of infrared beam. On the other hand, very thin films may not coat the reflection element with a homogeneous layer necessary to prevent artifacts due to strong protein adsorption on the bare ZnSe surface. The properties of the film depended strongly on the polymer concentration and composition of the solvent. Higher cellosolve concentrations in the solvent led to inhomogeneous coatings of the reflection element due to bad spreading of the solution on the ZnSe surface; lower ethanol concentrations in the solvent resulted in the formation of films with orange peel surfaces due to fast film drying.

Unlike the polymer films of hydrophobic polymers, such as polystyrene, also used in similar ATR experiments earlier [15], the water-swollen hydrophilic poly(HEMA/MA) films were generally unstable in aqueous solutions. The stability of films in aqueous solutions was assessed from the intensity of spectral bands of the polymer, e.g., of the carbonyl band at 1725 $cm^{-1}$. A decrease observable both in solution and after repeated drying the film indicated a decrease in polymer mass by partial dissolution. The recovery of intensity of polymer bands after drying some of the films indicated that water penetrated between the polymer film and the reflection element due to insufficient film adhesion to ZnSe surface. In such cases, the decrease in intensity of polymer spectral bands observed in solution was accompanied by the increase of water spectral bands (3400 $cm^{-1}$, 1640 $cm^{-1}$). To improve stability of poly(HEMA/MA) films in aqueous solutions, the films coated on the reflection element were additionally covalently crosslinked by UV irradiation. Optimum film stability was reached using a solution prepared from a polymer formulation obtained after 6 days' red-ox initiated polymerization. A decrease of about 1.1% in the poly(HEMA/MA) spectral bands was observed after first 5 h of incubation the film with PBS, probably due to the washing impurities and residual monomers. No significant additional decrease was observed in the next 5 h. A longer polymerization time resulted in lower concentrations of residual monomers in the formulation that were not sufficient for subsequent crosslinking. About 50% of polymer mass was washed out with PBS from a UV-crosslinked film prepared from the formulation polymerized for 13 days. On the other hand, formulations polymerized for less than 4 days were not viscous enough for the spin-coating probably due to a low content of high molecular weight polymer.

The deposition of collagen I on the surface of poly (HEMA/MA) film from 0.005 M HCl and phosphate buffer pH 6.5 (PB) observed in situ by IR ATR is shown in Fig. 3, curves 1 and 2, respectively. There was no observable deposition of collagen from acid solutions below pH 5. In

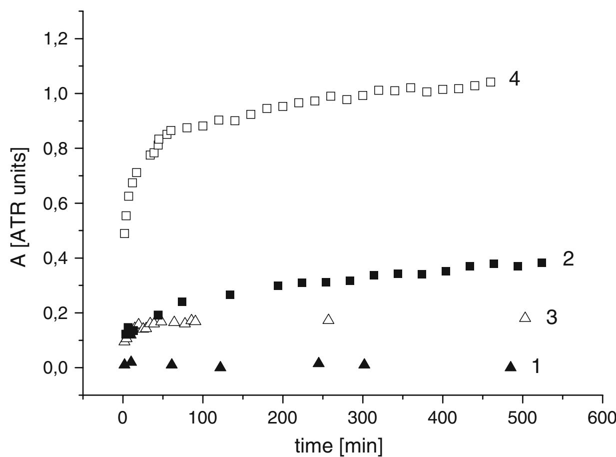


**Fig. 3** The deposition of collagen I from 50 μg/ml solutions on the surface of poly(HEMA/MA) film from 0.005 M HCl (curve 1) and from phosphate buffer pH 6.5 (curve 2), and on polystyrene film from 0.005 M HCl (curve 3) and from phosphate buffer pH 6.5 (curve 4) observed in situ by measuring IR ATR (A is the integral intensity of the protein band at 1548 $cm^{-1}$)

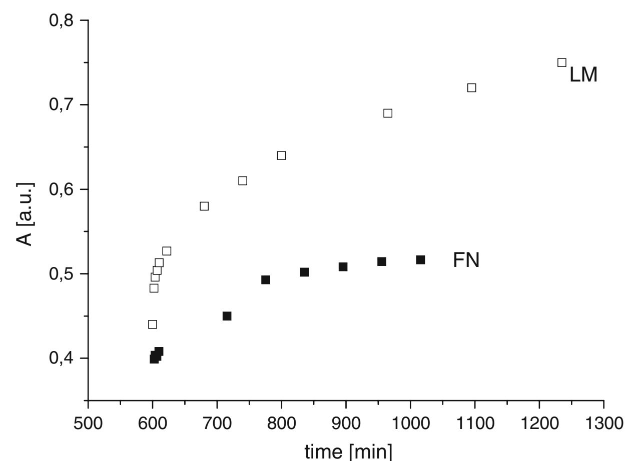


**Fig. 4** The attachment of laminin (LM) and fibronectin (FN) from 50 μg/ml PBS to a collagen I layer initially deposited on poly(HEMA/MA) film by incubation with collagen I solution, 50 μg per 1 ml of phosphate buffer pH 6.5, for 4 h; Observed in situ by measuring IR ATR (A is the integral intensity of the protein band at 1548 $cm^{-1}$)

contrast, collagen adsorbed from 0.005 M HCl on a polystyrene film (Fig. 3, curve 3). This was due to the fact that collagen can adsorb on polystyrene by hydrophobic interactions forming a monomolecular layer [15]. Collagen I used here completely dissolved in 0.02 M acetic acid, but it was only partially soluble at pH 6.5. The adsorption of collagen aggregates present in solution and/or the adsorption associated with precipitation of collagen at the surface were probably responsible for the high collagen amount deposited from PB at pH 6.5 without reaching marked saturation (Fig. 3, curves 2 and 4). An amount of collagen deposited on poly(HEMA/MA) in the experiment shown in Fig. 3, curves 2, was estimated to be 0.65 $\mu g/cm^2$.

Laminin and fibronectin adsorbed fairly well from PBS solution on the collagen layer deposited on poly(HEMA/MA) surface (Fig. 4). There was no adsorption of fibronectin on unmodified poly(HEMA/MA). The amount of laminin adsorbed on unmodified poly(HEMA/MA) was only 5% of that adsorbed on the collagen layer. Insolubility of the deposited collagen layer at pH 7.4 and a specific interaction of collagen with LM or FN provided long-term stability for these layers in PBS.

Collagen I was deposited from 0.005 M HCl on poly (HEMA/MANa) hydrogel plates swollen with PB, pH 7, so that the diffusion of PB from the hydrogel would increase pH at the interface with collagen solution. The increase in pH was assumed to induce deposition of collagen I on the hydrogel surface on which FN and LM could be subsequently attached. Unfortunately, IR reflection techniques available for our experiments could not be used for direct confirmation of protein deposition on the hydrogel plates. However, a considerable effect of the surface modifications on cell-seeding indicated that the proteins were deposited.

After seeding the MSCs and astrocytes on the modified poly(HEMA/MA) hydrogels, the cells started attaching and spreading onto the surfaces. Figure 5 shows the images of MSC and astrocytes cultivated 4 days after seeding them on modified poly(HEMA/MA) hydrogel surfaces. The results evaluated by pictures of four samples for each surface modification are characterized qualitatively in Table 1. The average surface area of single cell measured in mesenchymal stem cells (SMS) and astrocytes on modified hydrogel surfaces was evaluated by fluorescence microscopy on 4th day after seeding. In agreement with the results of experiments with various cells published so far, very poor adhesion and no proliferation of MSC and astrocytes were observed on unmodified poly(HEMA/MA) hydrogel. Coating hydrogel surface with a collagen I layer improved the cell growth markedly. However, a significant improvement in cell growth was reached by attachment of fibronectin and laminin on the collagen layer.

## 4 Conclusions

Collagen I soluble only at low pH could not be deposited on of poly(HEMA/MA) hydrogels by adsorption from acid solutions. The decreased collagen solubility at higher pH made it possible to deposit collagen I at pH 6.5 on the hydrogel on which fibronectin and laminin could be subsequently attached by adsorption from PBS. The deposited protein assemblies were stable in PBS at pH 7.4. The increase in pH of collagen solution at the surface of

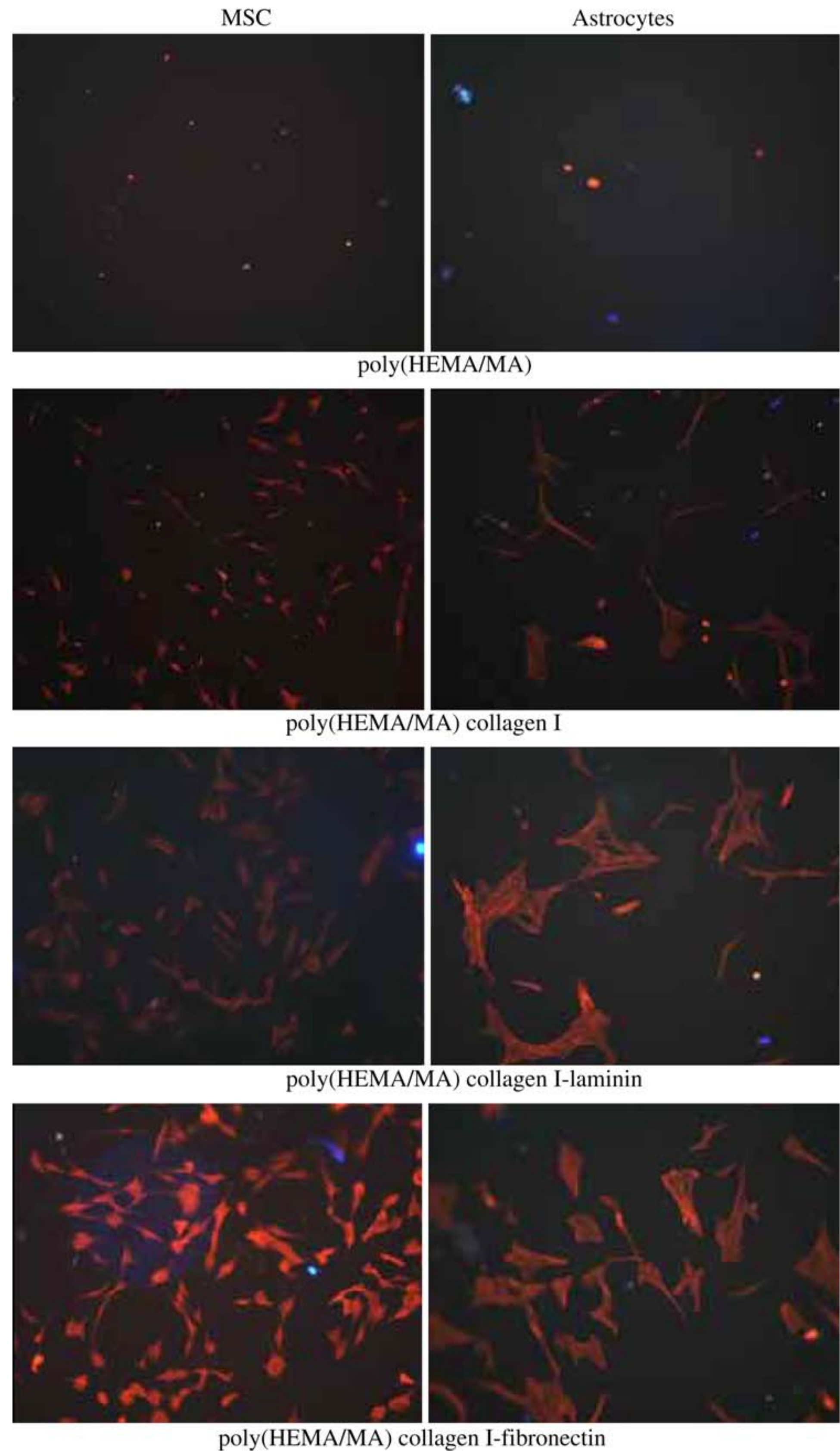


**Fig. 5** Cells on modified poly(HEMA/MA) hydrogel surfaces 4 days after seeding. MSC, mesenchymal stem cells obtained from the bone marrow, stained with phalloidin

**Table 1** The average surface area of single cell measured in mesenchymal stem cells (MSC) and astrocytes on modified hydrogel surfaces evaluated by fluorescence microscopy on fourth day after seeding

| Surface | MSC ($\mu m^2$) | Astrocytes ($\mu m^2$) |
|---|---|---|
| poly(HEMA/MA) | 3.9 ± 0.2 | 9.0 ± 0.9 |
| poly(HEMA/MA) collagen I | 23.1 ± 2.3 | 93.0 ± 12.7 |
| poly(HEMA/MA) collagen I-laminin | 69.0 ± 9.7 | 239.2 ± 42.2 |
| poly(HEMA/MA) collagen I-fibronectin | 49.3 ± 3.8 | 169.5 ± 16.9 |

The data for a surface were calculated from measuring three samples, 16 cells in each

poly(HEMA/MA) hydrogel swollen with a buffer of pH 7.6 was used for deposition of collagen I on the surface. The modification with collagen and laminin or fibronectin improved considerably the adhesion and growth of cells on the originally cell-non-adhesive hydrogel surface.

A collagen coating on flat surfaces is usually prepared by a short exposition of a thin layer of acid collagen solution deposited on the surface to ammonia vapors. Unlike this technique, the approach used in this work can be applied to coating three-dimensional hydrogel objects of any shape including coating the inner surfaces of porous scaffolds. Other EMC proteins can be attached to such surfaces without using chemical reagents interacting with the bulk hydrogel.

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