

***N*-(2-Hydroxypropyl) Methacrylamide Based Cryogels – Synthesis and Biomimetic Modification for Stem Cell Applications**

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Summary

The design of favorable mechanical properties and suitable surface modifications of hydrogels in order to stimulate specific cell response is a great challenge. *N*-(2-Hydroxypropyl) methacrylamide (HPMA) was utilized to form macroporous cryogel scaffolds for stem cell applications. Furthermore, one group of scaffolds was enhanced by copolymerization of HPMA with methacryloyl-GGGRGDS-OH peptide in an effort to integrate biomimetic adhesion sites. The cryogels were characterized by stiffness and equilibrium swelling measurements as well as by scanning electron microscopy. Cell culture experiments were performed with human adipose-derived stem cells and substrates were found completely non-toxic. Moreover, RGDS-enriched cryogels supported cell attachment, spreading and proliferation, so they can be considered suitable for designed aims.

Key words

Hydrogels • Cryogels • Stem cells • Scaffolds • Mechanical properties

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Introduction

Hydrogels are three-dimensional hydrophilic polymer networks capable of absorbing high amounts of

water or biological fluids. Due to their swelling capacity and soft consistency, they more closely simulate natural living tissue than any other class of synthetic biomaterial (Ko *et al.* 2013, Hoffman 2012, Peppas *et al.* 2000). Since the pioneering soft contact lens application that dates back to 1960 (Wichterle and Lim 1960), these materials have been used as drug delivery systems (Bertz *et al.* 2013, Xinming *et al.* 2008), wound-healing matrices (Gong *et al.* 2013, Heilmann *et al.* 2013, Li *et al.* 2011) and for cell cultivation (Cavalcanti *et al.* 2013, Matricardi *et al.* 2013, Saha *et al.* 2008, Studenovska *et al.* 2008, Reis *et al.* 2012, Kubinová *et al.* 2013, Radhakrishnan *et al.* 2014).

A wide range of polymers and diverse compositions has been used to fabricate hydrogels (Buwalda *et al.* 2014, Drury and Mooney 2003, Hoffman 2012, Kirschner and Anseth 2013). They can be both natural and synthetic polymers such as acrylamide (Kuyukina *et al.* 2009, Plieva *et al.* 2007, Saha *et al.* 2008), poly(*N*-isopropylacrylamide) (Buwalda *et al.* 2014, Koetting *et al.* 2015), chitosan (Reis *et al.* 2012), polysaccharides (Li *et al.* 2011, Matricardi *et al.* 2013) or collagen (Lewis *et al.* 2012). A class of polymers based on monomer *N*-(2-Hydroxypropyl) methacrylamide (HPMA) was utilized as a smart polymer in many applications including tissue engineering, drug delivery and macromolecular cancer therapy due to its considerable advantages, such as non-immunogenicity and non-toxicity. Moreover, a racemic mixture of DL

HPMA can be refined through crystallization to give the monomer higher purity (Ulbrich and Subr 2010, Kopecek and Kopeckova 2010). The fundamental requirements for biomaterials intended for stem cell applications consist in the design of favorable mechanical properties and suitable surface modifications to stimulate a specific cell response (Armentano *et al.* 2010). The greatest challenge is the tuning of mechanical properties of hydrogels in accordance with the desired type of tissue (Reilly and Engler 2010). The correlation between biomaterial stiffness and cell behavior has been demonstrated in *in vitro* studies of various engineered tissues, e.g. glial and neural tissues ($E' \sim 100$ Pa) (Saha *et al.* 2008), muscle ($E' \sim 10$ kPa) (Discher *et al.* 2005) and bone tissue ($E' > 30$ kPa) (Reilly and Engler 2010).

The ways of reaching these aims typically include tuning the material structure by introducing pores and cell binding ligands (Chen *et al.* 2011, He *et al.* 2007, Hsieh *et al.* 2007, Lozinsky *et al.* 2003, Noppe *et al.* 2007). In the case of chemically crosslinked hydrogels, a matrix architecture can be created by conducting polymerization in the presence of a porogen (Michalek *et al.* 2005, Sun *et al.* 2014). The additional incorporation of biomimetic motifs provides the possibility to stimulate various cell responses. Bioactive molecules derived from extracellular matrix proteins or other functional biomolecules are commonly used to enable e.g. the attachment of cells to material surfaces (Hacker *et al.* 2003, Kubinová *et al.* 2010, Rahmany and Van Dyke 2013, Yu *et al.* 2005). One of the commonly-used “golden standard” ligands is the fibronectin-derived RGD peptide (Adelöw *et al.* 2008, Ko *et al.* 2013).

Cryogels are a group of macroporous hydrogels that allow the user to adjust the before-mentioned parameters. Moreover, their preparation is usually low-cost and unpretentious since the material is produced under freezing conditions and crystals of the frozen solvent act as a porogen (He *et al.* 2007, Hsieh *et al.*, 2007, Lozinsky *et al.* 2003, Lozinsky 2014, Noppe *et al.* 2007). The main advantage of this method is the production of gels with a high swelling capacity and a spongy structure while preserving good mechanical stability (Gun'ko *et al.* 2013, Fromageau *et al.* 2007, Topuz and Okay 2009).

The present work is focused on investigating the mechanical properties of HPMA cryogels during alteration of the initial reaction mixture composition. For these purposes, the *N*-(2-Hydroxypropyl) methacrylamide as a monomer and poly(ethylene glycol) dimethacrylate

as a crosslinker were used. To our current knowledge, the HPMA was never yet polymerized as cryogel. Furthermore, the methacryloylated RGDS-peptide was used as a comonomer to enrich materials with biomimetic sites. The functionality of the modified hydrogels was evaluated by seeding and growing adipose-derived stem cells within the gels.

Material and Methods

Materials

Tetramethylethylenediamine (TEMED), ascorbic acid, poly(ethylene glycol) dimethacrylate (PEGDMA) and dichloromethane (DCM) were purchased from Sigma-Aldrich (Czech Republic), *N*-(2-hydroxypropyl) methacrylamide (HPMA) was prepared according to a method mentioned elsewhere (Kopecek and Bazilova 1974) and *N*-Methacryloyl-NH-GGGRGDSG-OH (RGDS) peptide was synthesized according to a previously-published procedure (Studenovska *et al.* 2010). The cryogelation was performed in a Ministat 2400 cryostat (Huber, Offenburg, Germany). The sample morphology was evaluated by scanning electron microscopy (SEM) on a Vega Plus TS 5135 (Tescan, Czech Republic). The stiffness of the hydrogels was characterized by measuring their compressive modulus using an Instron 6025R5800 (Norwood, MA, USA) universal tester.

Collagenase Type IV, DMEM (Gibco, Life Technologies, Paisley, UK), FBS, Penicillin/Streptomycin (PAA Laboratories GmbH, Pasching, Austria) and gelatin were used to cultivate cells.

Acridine orange (A6014, Sigma-Aldrich) and ethidium bromide (46065, Sigma-Aldrich) were used for the biological live/dead analysis. Cell morphology was analysed by staining cytoskeletal actin and chromatin using Phalloidin Rhodamine (R415, Life technologies, Czech Republic) and 4,6-diamidino-2-phenylindole (DAPI, 32670, Sigma-Aldrich, Czech Republic). Microscopy observations were performed using Nikon Eclipse Ti-E system, equipped with an 20× objective (Nikon, Prague, Czech Republic) and a Cool Snap HQ2 CCD camera (Photometrics), controlled by NIS-Elements imaging software (Nikon, Prague, Czech Republic), Confocal Laser Scanning Microscopy system Fluoview500 (Olympus C&S Ltd., Prague, Czech Republic) equipped with an 40× objective for morphological analysis of cells by SEM on a Vega (Tescan Orsay Holding, Brno, Czech Republic).

Formation of HPMA based cryogels

Cryogel formation was carried out in 5-ml glass vials. Two types of crygel based on N-(2-hydroxypropyl)methacrylamide (HPMA) were prepared. For the first type, appropriate amounts of HPMA and PEGDMA as a crosslinking were dissolved in Q-water to obtain 7.5 w/v% solutions with different a range between the monomer and the crosslinker. The second type of gel was modified with an RGD-peptide. In this case, methacrylated RGD peptide was used as a comonomer at 3 w/w% for all of the solids and the crosslinker concentration was 2.4 mol%.

A portion of the initial solution was added to each vial; the solution was then purged with nitrogen and brought to a temperature near zero followed by the TEMED and subsequent addition of APS (0.02 g/ml) for a final concentration 2 w/w% of all the solids. The vials were placed in a temperature of -15 °C for 3 h. Then, all vials with frozen solutions were kept at -18 °C for 12 h. After freezing, the samples were thawed at room temperature and rinsed with distilled water. The washed samples were dried in a freeze-dryer until a constant weight was reached.

Water uptake measurement

The dried hydrogels were weighed and then swollen at room temperature in Q-water until equilibrium was reached. At least five measurements were performed for each hydrogel sample, and the average weight of the swollen hydrogels was calculated. The value of equilibrium water content was determined using equation 1.

$$S = \frac{m_s - m_d}{m_d} \frac{1}{\rho}, \quad (1)$$

where

m_s - weight of the gel with equilibrium water content, g;

m_d - weight of the dry gel, g;

ρ - solvent density, g/ml.

Scanning electron microscopy

A Vega TS 5135 scanning electron microscope (Tescan, Czech Republic) was employed to investigate the morphology of the hydrogels. Before observation with the electron microscope, the hydrogel samples were sputtered with a Pt layer (SCD 050 vacuum sputter coater; Leica). SEM micrographs were obtained using secondary electron imaging at 30 kV.

Material stiffness evaluation

The studied hydrogels were subjected to compressive testing. The stiffness of the hydrogels was characterized by the compressive modulus of elasticity. Cylindrical specimens having an h/D (height-to-diameter) ratio of $\sim 1/3$ were subjected to compressive loading using an Instron 6025R5800 universal testing machine. The specimens had a diameter of approximately 16.5 mm and were deformed at a constant test speed of 1.0 mm/min at ambient temperature. During the test, the specimens were immersed in distilled water. The modulus of elasticity was calculated from the linear portion of the stress-strain diagram by dividing the change in stress, $\sigma_2 - \sigma_1$, by the corresponding change in strain, $\varepsilon_2 = 0.10$ minus $\varepsilon_1 = 0.05$.

Seeding of cells on materials

Adipose-derived stem cells were isolated from adipose tissue by centrifugation and collagenase extraction based on Zuk's method (Zuk *et al.* 2002).

Briefly, adipose tissue was digested with 0.1 % Collagenase Type IV for 30 minutes at 37°C. Following enzyme activity neutralization by DMEM-F12 with 10 % FBS, the cells were separated by centrifugation at 600 g. The pellet was re-suspended in a cultivation medium (10 % FBS, 0.5 % Penicillin/Streptomycin in DMEM Glutamax) and propagated for 5 passages in a culture dish coated with 0.01 % gelatin. Thin layers of cryogels were cut into 3 x 3 mm working pieces and washed three times with a 0.1 M phosphate buffer. Adipose-derived stem cells were released from the cultivation plastic dish by trypsin digestion and seeded on cryogels in a 500 μ l suspension with a concentration of 6.10^5 cells/ml and incubated in a 5 % CO₂ atmosphere at 37 °C for 24 h.

Cell adhesion evaluated by SEM

Hydrogel samples were fixed in 3 % glutaraldehyde (Polysciences, Inc., Warrington, USA) dissolved in a 0.1 M phosphate buffer for 1 h at room temperature. Subsequently, samples were washed three times for 10 min in a 0.1 M phosphate buffer, dehydrated in ascending ethanol grade (30, 50, 70, 80, 96, 100 %) and dried in a Critical Point Dryer (CPD 030, Balzers Union Limited, Balzers, Liechtenstein) using liquid carbon dioxide. Samples were sputtered with gold in a Sputter Coater (SCD 040, Balzers Union Ltd, Balzers, Liechtenstein) and examined by SEM (Vega, Tescan Orsay Holding, Brno). SEM micrographs were obtained using secondary electron imaging at 15 kV.

Results and Discussion

All polymerization experiments were carried out at $-15\text{ }^{\circ}\text{C}$. The ammonium persulfate / N,N,N,N-tetramethylethylenediamine system was used for the initiation. The solvent uptake by the samples was measured in water. The inner structure (morphology) of the samples was analyzed by SEM and the stiffness of the hydrogels was measured by compression testing.

In our previous work, it was shown that mechanical properties, porosity and swelling were dependent on the method of hydrogel preparation. However, due to irradiation, additional crosslinks in the polymer network appeared and there was also a potential influence of the irradiation on the structure of the RGD peptide. In this work, we therefore used HPMA as a monomer, methacryloyl-GGGRGDS-OH peptide as a comonomer, and PEGDMA as a crosslinker. The

substantial initiation system was performed with TEMED/APS to overcome these limitations by using e-beam initiation conditions (Golunova *et al.* 2015).

The approach of hydrogel functionalization with biomimetic ligands, which was necessary for cell adhesion, was also changed. Previously-used click reactions are complicated in the case of methacrylic comonomer application due to the absence of a water soluble methacrylic derivative with alkyne functionality. Therefore, a water soluble methacrylated RGD-peptide was prepared. The next advantage in this approach was the elimination of click reaction catalyzer, Cu^{2+} . Although we have proven the concentration level of applied Cu^{2+} nontoxic in contact with cells (Proks *et al.* 2012, Golunova *et al.* 2015), it still gives us better options for making biomimetic modifications. The peptide concentration for the specific cell response (e.g. adhesion) was showed to be sufficient by 3% (w/w) (Studenovska *et al.* 2010), thus it was applied.

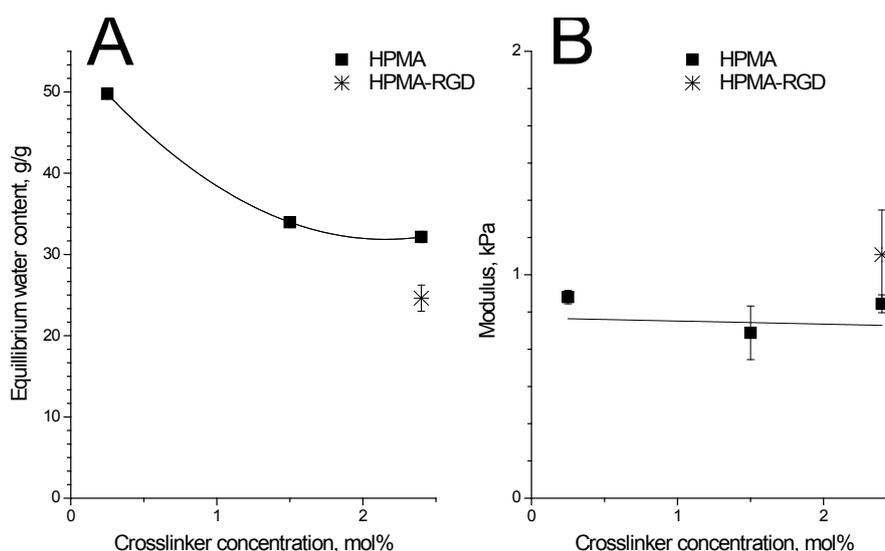


Fig. 1. Dependence of the equilibrium water content (**A**) and compressive modulus (**B**) on crosslinker concentration. The total monomer concentration was 7.5 wt%, the crosslinker (PEGDMA) concentration was 2.4 mol%, 1.4 mol%, 0.25 mol% respectively and the temperature was $-15\text{ }^{\circ}\text{C}$. In the case of HPMA-RGD gels, the crosslinker concentration was 2.4 mol%.

Elasticity and equilibrium water content

The equilibrium water content values decreased with an increasing concentration of crosslinker (Fig. 1A). It can be assumed that a lower crosslinker concentration would lead to fewer crosslinks and allow polymer chains to be more flexible and to be able to swell a higher amount of water. The swelling values of the RGD-modified gel were lower probably due to the extra bonds that could appear between the molecules of the peptides.

Contrary to this, the influence of the crosslinker concentration on stiffness was found to be very low, although the introduction of the peptide into the initial

composition of HPMA gels slightly increased hydrogel stiffness (Fig. 1B). That happened probably due to the extra hydrogel crosslinking between the peptide molecules through electrostatic interaction.

Previously reported poly(acrylamide) gels had a stiffness around 3 kPa (Golunova *et al.* 2015) and in comparison with the recent results, it can be assumed that the main effect on hydrogel stiffness was given by the main comonomer type. Therefore, we hypothesise, the stiffness regulation can be achieved by variation of the main monomer concentration, combination of different monomers and their ratio.

Morphometry of materials by SEM

SEM was used to evaluate the porosity of the cryogels and to examine the effects of the peptide's incorporation into the pore structure (Fig. 2). Both types of cryogels showed an interconnected macroporous

structure and the diameter of pores was measured in the range of 50-300 μm . It was also observed that the porous structure was not changed if the RGD peptide was used as a comonomer.

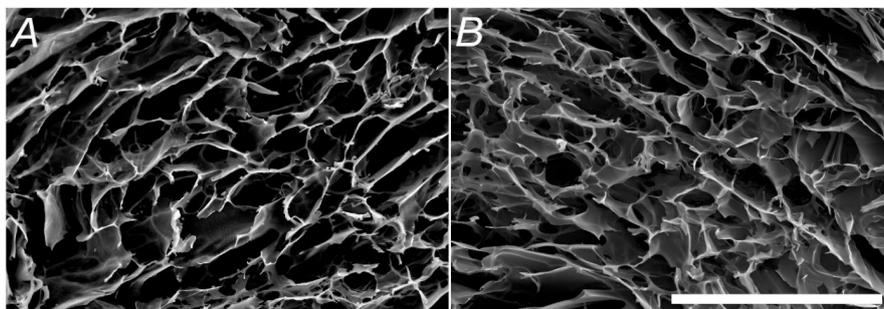


Fig. 2. Scanning electron micrographs of hydrogels obtained using different initial compositions. **(A)** An HPMA-based cryogel without an RGD-peptide and **(B)** with an RGD-peptide. The porous structure was comparable without any apparent morphological differences. The scale bar is 500 μm .

Evaluation of material biocompatibility and cell morphological response

Evaluating the essential characteristics of these scaffolds include an analysis whether the materials and their degradation products are not potentially toxic to cells and subsequently how the cells interact with gels.

Firstly, it is necessary to assess the polymer functionalization with adhesion ligands and their physical contact with cells on the surface, and then we must determine the influence of the material's stiffness and how this influences cell morphology and distribution.

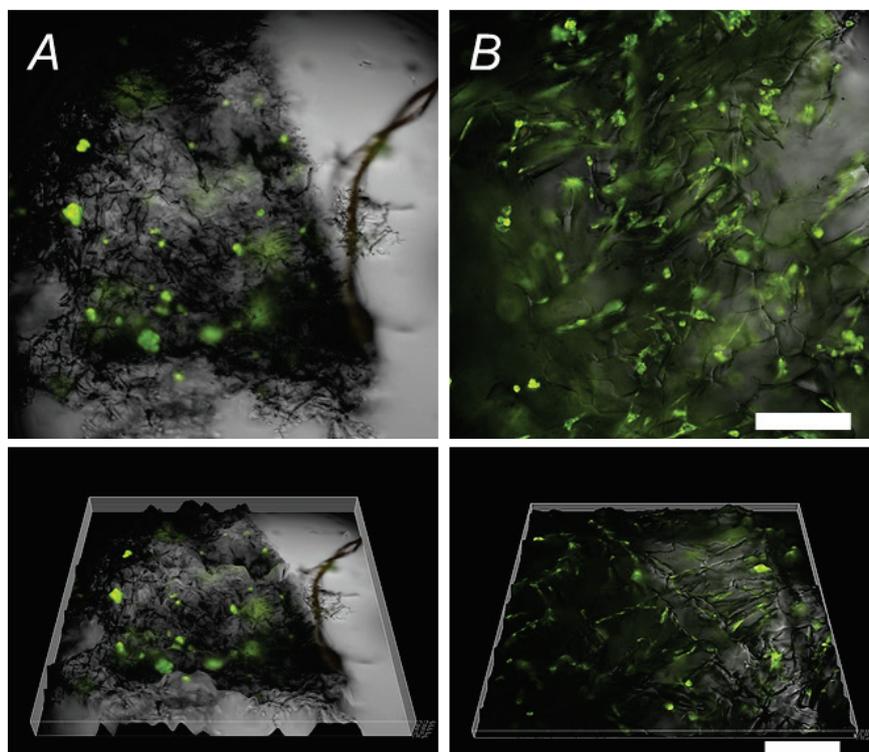


Fig. 3. Live/dead assay of ADSCs seeded on hydrogels with or without surface modification by the RGD peptide. Viable cells were labeled with acridine orange (green) and dead cells were stained by ethidium bromide (red) and were rarely observed. **(A)** HPMA-based cryogel without an RGD-peptide and **(B)** with an RGD peptide. The scale bar is 200 μm .

In this work, cells isolated from human adipose tissue were used, because they more closely approximate noncancerous human stem cells and are accessible for applications in clinical practice. Adipose-derived stem cells (ADSC) were homogeneously seeded within polymerized hydrogels with and without surface modification.

Microscopic observations of cells stained with live/dead assay displayed viable ADSCs (green) throughout both types of hydrogel scaffolds after 24 h cultivation, while there was only an occasionally appearance of dead (red) stained cells. Therefore, none of the materials induced cell death, which confirms their non-toxic nature (Fig. 3).

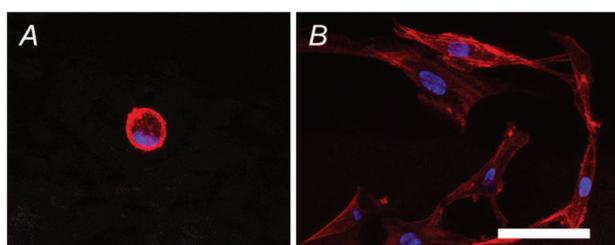


Fig. 4. Actin cytoskeleton (red) and nuclei (blue) staining of human adipose-derived stem cells. ADSCs attached and spread only to RGD modified hydrogels (**B**), ADSCs encapsulated in unmodified hydrogels (**A**) formed cell clusters without attaching to any material. The scale bar is 50 μm .

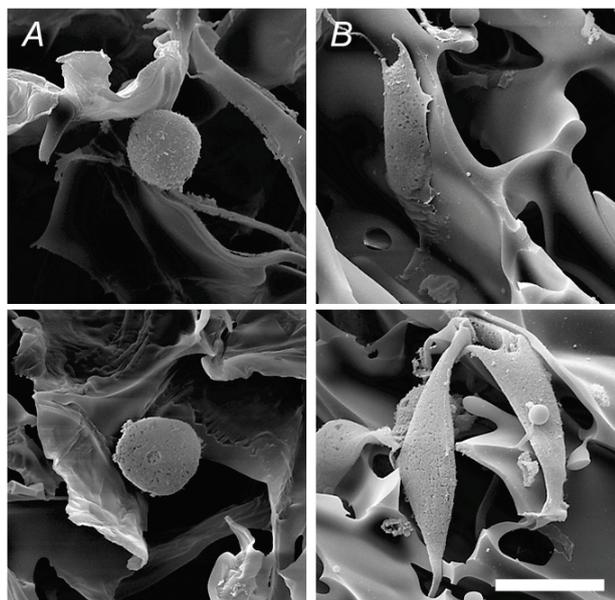


Fig. 5. Scanning electron microscopy of adipose-derived stem cell attachment and spreading on hydrogels (**A**) without modification and (**B**) with RGD peptide incorporated into the HPMA based cryogel. The focal adhesions of the cells are obvious when they contact modified hydrogels. The scale bar is 20 μm .

Using fluorescence microscopy on the actin cytoskeleton, it was observed that adipose-derived stem cells seeded on non-functionalized hydrogels formed spheres and cell clusters with minimal interaction with the material surface. The presence of an integrin-binding motif RGD was necessary for ADSC attachment, and the cells had regularly-shaped nuclei and formed strong microfilaments that spread both inside and across the material pores (Fig. 4).

The detailed morphological observations were complemented by SEM (Fig. 5). Cells seeded on unmodified hydrogels were round-shaped and formed cell clusters without any notable interaction between the cells and the material. On the other hand, RGD-modified materials supported cell attachment, allowed cells to fully spread on their surface and connect with other cells. The ADSCs had the same protruded cell shape as the cells growing under standard culture conditions, had regular microvilosity on the cellular surface and expressed visible contact with the supporting hydrogel material.

Conclusions

The soft cryogels were prepared by the free radical polymerization of the *N*-(2-Hydroxypropyl) methacrylamide as monomer and poly(ethylene glycol) dimethacrylate as the crosslinker. The variation of the crosslinker concentration affected swelling properties, but not hydrogel elasticity. Incorporating the methacryloyl-GGGRGDSG peptide in the reaction mixture allowed for biomimetic modification of the cryogels.

All the materials supported the viability of cells, moreover incorporating RGD peptide positively influenced the attachment and regular morphology of human adipose-derived stem cells during their cultivation. These results confirm the hydrogel biocompatibility and potential for further development of cryogels toward three-dimensional stem cell supports with capabilities for adjusting substrate elasticity and functionalization by bioactive molecules.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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